PCT/DK04/000822

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

Kongeriget Danmark

Patent application No.: PA 2003 01759

Date of filing: 28 November 2003

Applicant:
(Name and address)
Københavns Universitet
Det sundhedsvidenskabelige fakultet
Tech Trans Enheden
Blegdamsvej 3
DK-2200 København N
Denmark

Title: Plant disease resistance and SAR regulator protein

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.

Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

01 December 2004

Susanne Morsing

Plant disease resistance and SAR regulator protein

Field of the invention

The invention relates to broad spectrum disease resistance in plants and the identification, isolation and use of a novel regulator protein of systemic acquired resistance (SAR).

Background of the invention

Disease resistance is a primary determinant of crop yield, and monocultures of genetically uniform plants are particularly vulnerable to attack by pathogens to which they have low natural resistance. A key parameter in plant breeding is thus the selection of plants exhibiting broad range, as well as specific resistance to diseases caused by infectious agents, including viruses, bacteria and fungi. Pathogen attack can be perceived by a host plant through the specific recognition of pathogen-derived molecules. This in turn elicits a rapid, localised, hypersensitive response by the plant, in the form of rapid necrosis at the point of pathogen attack. The host-pathogen interaction also induces a plant immune response known as systemic acquired resistance (SAR), which provides long lasting protection against a spectrum of pathogens in the uninfected parts of the plant (Yang *et al.*, 1997, *Genes Develop.*, 11: 1621-1639). Induction of SAR is thought to rely on the release of one or more signal molecules, including salicylic acid (SA), at the site of infection and their movement throughout the plant via the phloem. Perception of this systemic signal by target cells leads to the coordinate expression of a subset of pathogenesis-related (PR) genes, which contribute to building and maintaining disease resistance. Exogenous application of SA appears to be sufficient to induce SAR and PR gene expression, while depletion of SA, by *in planta* expression of bacterial salicylate hydroxylase (NahG), suppresses SAR (Gaffney *et al.*, 1993, *Science* 261: 754-756).

Genetic screens, conducted in *Arabidopsis* to select mutants in the signal transduction pathway leading to SAR, have provided a fruitful approach to identify potential positive and negative regulators of SAR. Some mutants show enhanced disease susceptibility, either due to a failure to accumulate SA, for example *eds*1 (Falk *et al.*, 1999, *Proc Natl Acad Sci USA*, 96: 3292-3297), or a failure to perceive SA and induce PR gene expression, as exemplified by the *npr1* mutant (Cao *et al.*, 1997, *Plant Cell*, 88: 57-63). The *npr1* mutants (also known as the *nim1* non-inducible immunity mutant), carry mutations in a gene encoding NPR1 protein, which comprises ankyrin repeats that facilitate protein-protein interactions. NPR1 is believed to interact with basic leucine zipper transcription factors that bind and regulate expression from PR gene promoters (Zhang *et al.*, 1999, *Proc Natl Acad Sci USA*, 96: 6523-6528).

Other mutants, identified by genetic screening, display enhanced disease resistance. Lesion mimic mutants which constitutively express SAR and develop spontaneous necrotic lesions in the absence of pathogen challenge are common; however these may result from pleiotropic disruption of cellular homeostasis (Molina *et al.*, 1999, *Plant J.* 17: 667-678). Constitutive defence mutants (*cpr*) have also been found which show elevated SA levels and constitutive PR gene expression, without forming spontaneous necrotic lesions (Bowling *et al.*, 1994, *Plant Cell* 6: 1845-1857; Clarke *et al.*, 1998, *Plant Cell* 10: 557-569). PR gene expression in these *cpr* mutants is dependent on the SA signal.

Mutant screens have identified two negative regulator genes of SAR, namely *SNI1* and *MPK4*. *sni1* mutations, which cause enhanced SAR, are likely to regulate SA perception, since the *sni1* (suppressor of no-immunity) mutation can restore SAR in *npr1* mutants, which are otherwise unable to respond to SA application by inducing SAR (Dong *et al.*, 2001, *Novartis Foundation Symposium* 236: 165-173). The *Arabidopsis MPK4* gene encodes a

Mitogen-activated Protein kinase 4 (MPK4) that under non-pathogenic conditions, constitutively represses SAR. Mutations in the *MPK4* gene lead to increased SAR, as measured by enhanced SA levels and PR gene expression, and greater resistance to both bacterial and oomycete pathogens (Petersen *et al.*, 2000, *Cell* 103: 1111-1120). The expression of at least 16 genes, including 8 PR genes, is significantly increased in *mpk4* mutants, consistent with a constitutive SAR phenotype, while expression of certain jasmonic acid (JA)-induced genes is blocked. The constitutive SAR of *mkp4* mutants is dependent on SA, and is abolished by *in planta* expression of bacterial salicylate hydroxylase. The *mkp4 Arabidopsis* mutant is characterised by a dwarf habit, but the plants do not form spontaneous lesions. Mutants homozygous for both *mpk4* and *npr1-1* are dwarf and constitutively express PR genes and SAR as in *mpk4* mutants, while showing the SA hypersensitivity typical of *npr-1,* suggesting that MPK4 and NPR1 may be components of independent disease resistance pathways. Unlike NPR1, MPK4 appears to be involved in cross-talk between the JA- and SA-induced gene expression. While both MPK4 and NPR1 proteins regulate plant disease responses, they are believed to control the coordinate expression of different subsets of PR genes. Those PR genes regulated by MPK4 have been found to share similar *cis*-elements in their promoter sequences that may regulate their coordinate expression, but which are distinct from NPR1 regulated PR genes (Petersen *et al.*, 2000, *supra*). One of these elements, called a W-box, is a consensus binding-site for plant-specific WRKY transcription factors (Eulgem *et al.* 2000 *TIPS* 5: 199-206) that has been shown to act as a silencing element in the promoter of the PR1 gene (Lebel *et al.* 1998 *Plant J.*16: 223-33)

Several approaches are proposed to enhance the broad-spectrum disease resistance of crop plants. WO 9749822 describes the isolation of the *NIM1* gene, and its expression in transgenic plants in order to increase PR gene expression and thereby enhance SAR. WO 01/66755 and WO200053762

describe the isolation of various plant homologues of the *Arabidopsis NIM1* gene and their expression in transgenic plants to enhance SAR. Similarly, WO2000028036 describes transgenic plants expressing the *NPR1* gene conferring enhanced SAR. An alternative approach to increase SAR in plants is described in WO2001002574 and involves silencing expression of the gene encoding the SNI1 negative regulator polypeptide. Silencing or blocking the activity of MPK4, a second negative regulator of SAR, in order to enhance broad resistance to plant pathogens is disclosed in WO 01/41556.

It is generally recognised that wide spread use of pesticides is a standard agricultural practise which is to the detriment of the environment, and the accumulation of their residues in ground water is a serious man-made problem. Hence there is a strong desire throughout the world to reduce agricultural dependence on chemical pesticides, and to focus on enhancing the inherent resistance of plants to disease by breeding and genetic engineering. The production of crop plants with improved broad range resistance to plant pathogens relies on the identification of plant genes and their respective proteins products, whose expression determines the level and extent of immunity to pathogen attack. In particular plant genes which are components of one of more disease resistance signalling pathway, i.e., are involved in their regulation, can provide useful tools to control the timing or level of a given defence response. The value of this approach is clearly exemplified by the examples given above, where modulated expression of SAR regulatory genes in transgenic plants can enhance resistance to various pathogens. It is preferable to modulate the expression of a positive regulator of SAR, since techniques designed up-regulate gene expression in a transgenic plant are generally more effective than those required to achieve complete silencing of gene expression. It is particularly desirable that any improvement in pathogen resistance attained in the transgenic plant is not accompanied by the formation of lesions due to a spontaneous hypersensitive response, since this will be highly disadvantageous to both

the yield and quality of the crop. It is furthermore desirable to identify genes, which can be used to increase plant resistance to a wide range of natural pathogens, without impairing the plants ability to respond to and survive other predators or environmental stresses.

Summary of the invention

The present invention is based on the identification of a positive regulator protein of systemic acquired resistance (SAR) in plants. MKS1 is shown to be an integral component of the SAR signal transduction pathway, interacting with other components of the pathway and positively regulating SA synthesis and PR gene expression. Enhancing the expression of this plant regulator protein is shown to increase SAR in plants and to increase their resistance to pathogen attack.

Accordingly, the invention provides a transgenic plant having increased expression of a positive regulator of systemic acquired resistance (SAR) and enhanced disease resistance characterised by a transgene comprising a nucleic acid sequence encoding a MAP kinase substrate 1 (MKS1) polypeptide.

In one embodiment said MKS1 polypeptide has a primary amino acid sequence comprising a domain 1 with sequence: GPRPXPLSVXXDSHKIKKP and a domain 2 with sequence: PVIIYXXSPKVIHTXXXEFMXLVQRLTG, or conservatively modified variants thereof, wherein X refers to any amino acid residue.

In one embodiment the transgenic plant of the invention is characterised by a transgene having a nucleic acid sequence encoding a MKS1 polypeptide comprising an amino acid sequence selected from the group: SEQ ID No. 2, 6, 10, 14, 16, 20, 26, 27, 28 and conservative variants thereof.

In a further embodiment the transgenic plant of the invention, is characterised by a transgene comprising a nucleic acid sequence selected from the group: SEQ ID No. 1, 5, 9, 13, 15, 19, and conservative variants thereof, encoding said MKS1 polypeptide.

Furthermore the transgene of the transgenic plant of the invention may comprise a homologous promoter, or alternatively the transgene may be a chimeric gene comprising a heterologous promoter selected from the group: constitutive promoter, tissue specific promoter, and inducible promoter.

The transgenic plant of the invention includes either a dicotyledonous or a monocotyledonous plant and seed from the transgenic plant.

In a further aspect of the invention is provided a method for producing the transgenic plant of the invention, characterised by introducing an expression cassette, comprising the transgene encoding the MKS1 polypeptide, into a plant and selecting the transgenic plant and its progeny expressing said MKS1 polypeptide. Furthermore the invention encompasses a recombinant vector comprising said expression cassette and the introduction of said expression cassette into a plant through transformation or via a sexual cross with a transformed plant.

In another embodiment the invention provides a method for detecting increased expression of MKS1 polypeptide in the transgenic plant of the invention, characterised in reacting an anti-MKS1 antibody with a protein extract derived from said plant. Furthermore the invention encompasses both a polyclonal and a monoclonal anti-MKS1 antibody.

In another embodiment the transgenic plant of the invention may be used for the cultivation of a crop, wherein said crop encompasses plant biomass generated by the growth of a seed or seedling, and includes reproductive

parts, e.g. seed, caryopsis, cob, or fruit and vegetative parts, e.g. leaf and tuber.

In a further embodiment the transgenic plant of the invention is used in a breeding program, wherein a plant selected in the breeding program comprises the transgene having a nucleic acid sequence encoding a MKS1 polypeptide.

Brief description of the figures

Figure 1. *Arabidopsis* MPK4 and MKS1 interacting proteins.

A. Yeast two hybrid screening of an *Arabidopsis* cDNA Library with MPK4 as bait (BD fusion) identified MKS1 as an interacting prey (AD fusion), and screening with MKS1 as bait identified WRKY 25 and 33 as interacting prey (AD fusion). A directed two-hybrid assay (given in italics) between MKS1 as bait and MPK4 as prey, confirmed their interaction. Two-hybrid assays (in italics) between MKS1 as bait (BD fusion) and MPK3, 5, 6 and 17 as prey (AD fusion), as well as MKS1 or MPK4 as bait (BD fusion) and WRKY26, WRKY29 or WRKY25, WRKY33 as prey (AD fusion), respectively, showed no interactions. Yeast cells in the two hybrid screen were selected on the indicated nutrient depleted growth-media (-Histidine; - Leucine; - Adenine - Tryptophan) and assayed for β-galactosidase (β-gal) reporter gene activity.

B. ClustalW alignment of the amino acid sequence of *Arabidopsis* MKS1 (Acc.No:At3g18690) and homologues or orthologues from *Brassica oleracea* (Acc.No:BoBH544707 and BoBOHBT92TR + BOGQI24TF), *Glycine max* (Acc.No:GmBE020960), *Arabidopsis* (Acc.No:At1g21326; At1g68450, At2g41180, AtAL138658, At2g44340, AtT46022, At2g42140, AtAL390921) and *Oryza sativa* (Acc.No:OsCAD40925; OsBAC15955; OsAP004654, Os8360.t05160, Os8355.t00567, OsAP003260), *Nicotiana tabacum* (Ntacre169), *Zea mays* (Acc.No: ZmBM340911, ZmCC442903, ZmCC613160, ZmCC635639, ZmCC661221, ZmCC700850), *Medicago*

truncatula (Acc.No: MtAC143340.1). Identified and putative phosphorylation sites (SP) in MKS1 are indicated in italics. C-termini of the three MKS1 truncations and the Pep22 sequence are indicated above the MKS1 sequence. Aligned identical or equivalent amino acid residues are box-shaded. The consensus sequence of MKS1 is given below the alignment in bold, wherein Domain1 and 2 are underlined.

Figure 2. *In vitro* interaction and phosphorylation of MKS1 by MPK4.

A. 35S methionine-labelled MPK4 (lane 1), and its binding to MKS1-GST fusion protein (lane 3), but not to GST protein alone (lane 2), following separation by SDS-PAGE and detection by phosphoimager.

B. Phosphorylation assay with recombinant, full-length MKS1 (lane 1), C-terminal MKS1 truncations C1-C3, identified in Figure 1B (lanes 2-4), or positive control myelin basic protein (MBP, lane 5) and HA-tagged MPK4 immunoprecipitated from transgenic plants, analysed by SDS-PAGE and phosphoimager detection. Control phosphorylation assays were performed with HA-antibody immunoprecipitates of non-transgenic, wild-type (wt) plants (lanes 6-8).

C. Phosphorylation assay with recombinant, full-length MKS1 (lane 1); mutant full-length MKS1-S30A (lane 2), MKS1 C3-truncation (lane 3), or mutant MKS1-S30A C3-truncation (lane 4) and HA-tagged MPK4 immunoprecipitated from transgenic plants, and analysed as in (B).

D. Top: Phosphorylation assay with recombinant, full-length MKS1 alone (lane 1) or in the presence of increasing molar ratios of Pep22, indicated in Figure 1B (lanes 2-4) by HA-tagged MPK4, immunoprecipitated from transgenic plants, and analysed as in (B). Bottom: the phosphorylation assay (D. Top) was repeated with increasing molar ratios of a 22 amino acid peptide FLG22, as a negative control.

Figure 3. *In planta* interaction of *Arabidopsis* MKS1 and MPK4.

A. Immuno-detection of MKS1 in extracts of *E. coli* before (lane 1) and after (lane 2) IPTG induction, and in an extract of wild type *Arabidopsis* rosette leaves (lane 3) by polyclonal anti-Pep22 antibody in a Western blot (WB: pa-Pep22).

B. Immuno-detection of MKS1 immunoprecipitated (IP) with monoclonal anti-Pep22 (ma-Pep22) from wild type plant extract (lane 1) or control sample lacking plant extract (lane 2) by polyclonal antibody pa-Pep22 in a Western blot (WB: pa-Pep22).

C. Immuno-detection of HA-MPK4 by anti-HA antibody (Western blot; WB: ma-HA) in immunoprecipitates (IP) of *Arabidopsis* plant extracts using anti-Pep22 antibody, ma-Pep22 (lane 1); negative control monoclonal antibody, ma-Con (lane 2); or in a total protein plant extracts (lane 3), and a mock extract, comprising buffer and maPep22 antibody (lane 4).

Figure 4. Transgenic *Arabidopsis* plants with modified MKS1 expression

A. Immuno-detection of MKS1 with polyclonal antibody pa-Pep22 (Western blot; WB: pa-Pep22) in extracts of 35S-MKS1 transgenic *Arabidopsis* (lane 1), wild type *Arabidopsis* Ecotype Col (wt; lane 2) and RNAi-MKS1 transgenic *Arabidopsis* (lane 3) plants.

B. Growth phenotype of wild type *Arabidopsis* Ecotype Ler (wt), 35S-MKS1 transgenic *Arabidopsis* and *mpk4* mutant *Arabidopsis* plants.

Figure 5. Effect of MKS1 and MPK4 on expression of defense and wounding response genes in *Arabidopsis*

A. RNA blot detection of PR1 and MKS1 mRNA in *Arabidopsis* wild type Ecotype Ler (wt; lane 1), 35S-MKS1 transgenic (lane 2) and *mpk4* mutant (lane 3) plants.

B. RNA blot detection of VSP and WR3 mRNA accumulation in rosette leaves from nahG*mpk4* (lanes 1-4) and wild type Ecotype Ler plants (wt; lanes 5-8), at different times after wounding.

C. RNA blot detection of VSP mRNA in rosette leaves from wild type Ecotype Col (lanes 1 and 2) and RNAi-MKS1 plants (lanes 3 and 4), at 0h and 2h after wounding.

D. RNA blot detection of PDF1.2 mRNA in wild type *Arabidopsis* Ecotype Ler (wt; lane 1 and 2), RNAi-MKS1 (lane 3 and 4), and 35S-MKS1 transgenic (lane 5 and 6) plants, at 0h and 48 hr after methyl jasmonate (MeJA) treatment.

Figure 6. Properties of *Arabidopsis* plants with altered MKS1 expression

A. Salicylate levels (ng/g FT (fresh weight)) in leaves from 4-week-old 35S-MKS1 transgenic *Arabidopsis* and wild type (wt) plants grown in soil. Error bars show standard deviation of triplicates; absence indicates insignificant differences.

B. Pathogen virulence assay of 4-week-old wild-type *Arabidopsis* Ecotype Ler (wt), 35S-MKS1 transgenic *Arabidopsis* and *mpk4* mutant *Arabidopsis* plants inoculated with the virulent strain DC3000 of *Pseudomonas syringae* pv. *tomato* at a concentration of 1×10^5 colony-forming units per ml (CFU/ml). Values represent average and standard deviations of cfu extracted from leaf disks in three independent samplings.

C. Pathogen virulence assay of wild type *Arabidopsis* Ecotype Col (wt) and RNAi-MKS1 transgenic *Arabidopsis* plants. Values given are as in B.

D. GFP fluorescence detection of the GFP fusion proteins: MKS1-GFP, MPK4-GFP and GUS-GFP expressed in leaf mesophyll cells of transgenic *Arabidopsis* plants using confocal microscopy. Subcellular compartments indicated are: cytoplasm (cy); nucleus (nu).

Figure 7. A model of defense signaling in *Arabidopsis*, highlighting MPK4, MKS1, WRKY25 and WRKY33.

Detailed description of the invention

I. Abbreviations

GST: Glutathione-S-transferase

MKS1: Map Kinase Substrate 1

MPK4: Mitogen-Activated Protein Kinase 4

NahG: bacterial salicylate hydroxylase

PR gene /protein: Pathogen Related gene/protein

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

WB: Western Blot

WT: wild type

II. Definitions

***Agrobacterium*-mediated transformation**: is a technique used to obtain transformed plants by infection with *Agrobacterium tumefaciens.* During the transformation process the bacteria transfers a DNA fragment (T-DNA) from an endogenous plasmid into the plant genome. For transfer of a gene of interest the gene is first inserted into the T-DNA region of *Agrobacterium tumefaciens,* which is subsequently used for infection using the floral dip method according to Clough and Bent, 1998 in *Plant J* 16: 735-743.

Antibody: immunoglobulin protein that is produced in the body in response to immunisation with an antigen (for example MKS1 polypeptide or peptide fragment thereof), and that binds specifically to that antigen.

Breeding program: A breeding program encompasses the selection of progeny resulting from a sexual cross between parent plants. The sexual cross may be between defined parent plants or between a random population of parent plants. The progeny resulting from the cross are selected according to defined selection criteria including, but not limited to agronomic performance e.g. disease resistance, drought resistance, heat tolerance, yield, and the inheritance of a specific gene including a transgene.

cDNA: complementary DNA, comprising a 1st strand, complimentary to a mRNA molecule generated by reverse transcription, from which a 2nd complementary strand may be generated with a polymerase.

Chimeric gene: refers to a nucleic acid sequence, comprising a promoter operably linked to a second nucleic acid sequence containing an ORF or fused ORFs, which optionally may be operably linked to a terminator sequence. The promoter sequence is not normally operatively linked to the second nucleic acid sequence as found in nature, but is able to regulate transcription or expression of the second nucleic acid sequence. The second nucleic acid sequence codes for a mRNA and may be expressed as a protein.

Conservatively modified variant: refers to a polypeptide sequence when compared to a second sequence, and includes individual conservative amino acid substitutions as well as individual deletions, or additions of amino acids. Conservative amino acid substitution tables, providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another:

I: valine (V), leucine (L), isoleucine (I), methionine (M);
II: phenylalanine (F), tyrosine (Y), tryptophan (W);
III: arginine (R), lysine (K), histidine (H), glutamine (Q);
IV: aspartic acid (D), glutamic acid (E), asparagine (N), glutamine (Q);
V: alanine (A), serine (S), threonine (T).

In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variants". When referring to nucleic acid sequences, conservative modified variants are those that encode an identical amino acid sequence, (in recognition of the fact that codon redundancy allows a large number of different sequences to encode any given protein); or conservative modified variant; or a conservative modified variant having deletions or additions of a single amino acid or a small percentage of amino acids in the encoded sequence.

Crop: a crop encompasses plant biomass generated by the growth of a seed or seedling, and includes reproductive parts, e.g. seed, caryopsis, cob, or fruit and vegetative parts, e.g. leaf and tuber.

Dicotyledenous plant: flowering plant having two cotyledons in the seed.

Disease resistance: the term disease resistance indicates the ability of a plant to resist pathogen attack. 'As used herein "enhanced" resistance is a greater level of resistance to a disease causing pathogen by a transgenic or genetically modified plant, produced by the method of the present invention, as compared with a non-modified, control plant . In a preferred embodiment the level of resistance to a pathogen is at least 5%, preferably at least 10%, more preferably at least 20% greater than the resistance of a control plant.

Exon: protein coding sequence of a gene sequence.

Expression cassette: a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest, which is operably linked to termination signals. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components it heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or its progenitor by a transformation event.

Fusion protein: polypeptide read-through expression product of a gene comprising two or more protein coding sequences fused in frame.

Genetically modified plant: in terms of the present invention relates to a non-naturally occurring plant, whose genome has been artificially modified by genetic manipulation techniques, e.g., chemical mutagenesis, site-directed mutagenesis, homologous recombination (Terada *et al.* 2002 *Nature Biotech.* 20: 1030-1034) and transformation.

Genomic DNA: DNA sequences comprising the genome of a cell or organism.

Heterologous: a polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or from a different gene, or is modified from its original form. A heterologous promoter operably linked to a coding sequence refers to a promoter from a species, different from that from which the coding sequence was derived, or, from a gene, different from that from which the coding sequence was derived.

Homologous: a polynucleotide sequence is "homologous to" an organism or a second polynucleotide sequence if it originates from the same species, or gene. A homologous promoter refers to a gene promoter operably linked to the coding sequence of the same gene.

Homologue: is a gene or protein that is substantially identical to another gene's sequence or another protein's sequence.

Host cell: A prokaryotic or eukaryotic cell which may be transformed with an expression casette cloned in a vector. The host cell may be a bacterial (for example *Agrobacterium* spp, or *E.coli*) or plant cell (for example a monocotyledenous or dicotyledenous plant cell. The protein encoded by the expression cassette may be expressed and purified from the host cell.

Identity: refers to nucleic acid or polypeptide sequences that are the same or have a specified percentage of nucleic acids of amino acids that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the sequence comparison algorithms listed herein, or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to account for the conservative nature of the substitution. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch,

thus increasing the percent identity. Means for making these adjustments are well known to those skilled in the art.

Interacting: in terms of the present invention, relates to a physical interaction between two or more proteins, and their association for a duration sufficient to be detectable by known bioassays. For example, interacting proteins are detected by the yeast 2-hybrid screen and assay, and by co-precipitation with antibodies with affinity to one of the interacting proteins.

Intron: is a non-coding sequence interrupting a protein coding sequence within a gene sequence.

Isolated: in the context of the present invention an isolated protein (polypeptide) or an isolated nucleic acid molecule is a protein or nucleic acid molecule that, by the hand of man, exists apart from its native environment, and is therefore not the product of nature. The isolated protein or nucleic acid molecule may exist in a purified form or in a non-native environment such as, for example, a transformed host cell.

MAP kinase: mitogen-activated protein kinase, which acts downstream of other MAPK kinases, in reversible phosphorylation cascades to transduce extracellular signals into cellular responses (for example MPK4, 3, 5, 6, 17).

MKS1: MAP Kinase Substrate1 (MKS1) polypeptide is a positive regulator of SAR and enhances plant disease resistance. The primary amino acid sequence of MKS1 comprises domain 1 with sequence: GPRPXPLSVXXDSHKIKKP and domain 2 with sequence: PVIIYIVSPKVIHTXXSEFMXLVQRLTG, and conservatively modified variants thereof, wherein X refers to any amino acid residue. MKS1 is phosphorylated at one or more sites by a MAP kinase and it interacts with a transcription factor (for example a WRKY transcription factor). A MKS1 polypeptide includes a truncated or deleted fragment thereof that retains domain 1 and domain 2 sequences and the functional properties of being a positive regulator of SAR and enhancing plant disease resistance.

Monocotyledenous plant: includes, but is not limited to, barley, maize, oats, rice, rye, sorghum, and wheat.

Mutant: a plant or organism with a modified genome sequence resulting in a phenotype which differs from the common wild-type phenotype.

Native: as found in nature, and with respect to "native promoter" refers to a promoter operably linked to its homologous coding sequence.

RNA blot analysis: a technique for the quantitative analysis of mRNA species in an RNA preparation involving size separation of RNA by agarose gel electrophoresis, subsequent transfer of RNA from the gel to a nucleic acid binding membrane, and hybridisation of the membrane with sequence specific probes.

Operably linked: refers to a functional linkage; for example between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

ORF: Open Reading Frame, which defines one of three putative protein coding sequences in a DNA polynucleotide.

Orthologue: Homologous genes (or proteins) diverging concurrently with the evolutionary divergence of the organism harbouring them. Orthologues commonly serve the same function within the organisms and are most often located in a similar position on the genome.

PCR: Polymerase Chain Reaction is a technique for the amplification of a DNA polynucleotide, employing a heat-stable DNA polymerase and short oligonucleotide primers, which hybridise to the DNA polynucleotide template in a sequence specific manner and provide the primer for 5' to 3' DNA synthesis. Sequential heating and cooling cycles allow denaturation of the double-stranded DNA template and sequence-specific annealing of the primers, prior to each round of DNA synthesis. PCR is used to amplify a DNA polynucleotide employing the following standard protocol or modifications thereof:

PCR amplification is performed in 25 µl reactions containing: 10 mM Tris-HCl, pH 8.3 at 25°C; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.5 unit Taq polymerase and 2.5 pmol of each primer together with template genomic DNA (50-100 ng) or cDNA. PCR cycling conditions comprise heating to 94°C

for 45 seconds, followed by 35 cycles of 94°C for 20 seconds; annealing at X°C for 20 seconds (where X is a temperature between 40 and 70°C defined by the primer annealing temperature); 72°C for 30 seconds to several minutes (depending on the expected length of the amplification product). The last cycle is followed by heating to 72°C for 2-3 minutes, and terminated by incubation at 4°C.

Phosphorylated: in terms of the present invention relates to the phosphorylation of a protein, such as MKS1, by a protein kinase, such as a MAP kinase. Phosphorylation sites are commonly serine and/or threonine residues on the protein. Protein kinases act to regulate the activity of proteins by covalently attaching phosphate groups. The addition of this large charged group to the protein will usually result in changes in the target protein's conformation. These conformational changes typically result in changes in the protein's activity (either up or down) or association with other proteins. Protein phosphatases act in an opposite fashion and regulate proteins by removing phosphate groups that have been covalently attached to a protein (by a protein kinase).

Polynucleotide molecule: or "polynucleotide", or "polynucleotide sequence" or "nucleic acid sequence" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known analogues of natural nucleotides, which have similar binding properties as the reference nucleic acid.

Polypeptide: is any chain of amino acids, regardless of length or post-translational modification (for example glycosylation or phosphorylation).

Pathogenesis Related (PR) gene: is one that is activated or expressed in a cell of a plant in conjunction with pathogen attack and infection of the plant by a pathogen. Proteins encoded by PR genes include chitinase, extension (EXT1), PR1, PR5, Lipid transfer protein (LTP), β-1,3-glucanase (BGL2/PR2), β-1,3-glucanase (BGL3), glutathione-S-transferase (ERD11, PM24), LRR receptor kinase, monodehydroascorbate reductase, thionin,

osmotic, glycine-rich protein (GRR), phenylammonialyase (PAL), oxalate oxidase-like (GKP5).

Promoter: is an array of nucleic acid control sequences that direct transcription of an operably linked nucleic acid. As used herein, a "plant promoter" is a promoter that functions in plants. Promoters include necessary nucleic acid sequences near the start site of transcription, e.g. a TATA box element, and optionally includes distal enhancer or repressor elements, which can be located several 1000bp upstream of the transcription start site. A "tissue specific promoter" is one that specifically regulates expressed in a particular cell type or tissue, for example the promoter from the *Arabidopsis thaliana* RuBisCo small subunit gene NM_179480 [gi:30695946]. A "constitutive" promoter is one that is active under most environmental and developmental conditions throughout the plant, for example the 35S CaMV promoter (Acc.No:V00141, J02048), the *Arabidopsis* and maize *UBI1* gene promoter (Christensen *et al.*, 1992, *Plant Mol Biol* 18: 675-689), maize *ADH* gene promoter (Last *et al.* 1991 *Theor Appl Genetics* 81: 581-588), rice *ACT1* gene promoter (McElroy *et al.* 1990 *Plant Cell* 2: 163-172). An "inducible promoter" is one which is activated in the presence of a specific agent (the inducer), which may be a chemical compound or a physical stimulus such as heat or light. The chemical compound may be one that is not found in the plant in an amount sufficient to induce activation of the inducible promoter and transcription of the operably linked gene. Examples of inducible promoters include the ecdysone agonist inducible promoter (Martinez *et al.* 1999 *Plant J.* 19: 97-106), glucocorticoid agonist inducible promoter (Aoyama and Chua, 1997 *Plant J.* 11: 605-612), copper inducible promoter (Mett *et al.* 1993 *Proc Natl Acad Sci USA* 90: 4567-4571), ethanol inducible promoter (Caddick *et al.* 1998 *Nature Biotech* 16: 177-180), tobacco *WUN1* promoter (Seibertz *et al.* 1989 *Plant Cell,* 1: 961-968) and the disease-inducible *WRKY28* promoter (gi:17064157; Dong *et al.*, 2003 *Plant Mol Biol.*, 51: 21-37), and an inducible *MKS1* gene promoter may itself be used to direct expression in a *MKS1* coding sequence.

RACE/5'RACE/3'RACE: Rapid Amplification of cDNA Ends is a PCR-based technique for the amplification of 5' or 3' regions of selected cDNA sequences which facilitates the generation of full-length cDNAs from mRNA. The technique is performed using the following standard protocol or modifications thereof: mRNA is reverse transcribed with RNase H^- Reverse Transcriptase essentially according to the protocol of Matz *et al*, (1999) *Nucleic Acids Research* 27: 1558-60 and amplified by PCR essentially according to the protocol of Kellogg *et al* (1994) *Biotechniques* 16(6): 1134-7.

Real-time PCR: a PCR-based technique for the quantitative analysis of mRNA species in an RNA preparation. The formation of amplified DNA products during PCR cycling is monitored in real-time, using a specific fluorescent DNA binding-dye and measuring fluorescence emission.

Recombinant vector: a DNA molecule comprising sequences allowing self-replication in one or more host cells, e.g. *E.coli* or *Agrobacterium* spp., which may further comprise a heterologous chimeric gene, inserted into the vector DNA molecule. A recombinant vector, comprising a chimeric gene, may be transformed into a host cell for the purposes of expressing the chimeric gene. A recombinant vector comprising a chimeric gene also encompasses vectors for transformation of a plant, for example binary vectors.

Regulator: as referred to herein, is a protein which regulates another protein, pathway or response e.g. SAR, to either enhance or reduce the activity or level of said protein, pathway or response.

SAR: Systemic acquired resistance is a plant immune response which provides protection against a spectrum of pathogens in uninfected parts of a plant and is correlated with the expression of pathogenesis-related (PR) proteins, some with antimicrobial activity.

Sexual cross: refers to the pollination of one plant by another, leading to the fusion of gametes and the production of seed.

SMART consensus: represents the consensus sequence of a particular protein domain predicted by the Simple Modular Architecture Research Tool database (Schultz, J. *et al.* (1998)- *PNAS* 26;95(11):5857-64)

Southern hybridisation: A filter carrying nucleic acid (DNA or RNA) is prehybridized for 1-2 hours at 65°C with agitation in a buffer containing 7 % SDS, 0.26 M Na_2HPO_4, 5 % dextrane-suphate, 1 % BSA and 10µg/ml denatured salmon sperm DNA. Then a denatured, radioactively-labelled DNA probe is added to the buffer and hybridization is carried out over-night at 65°C with agitation. Unbound and non-specifically bound probe is then removed from the filter by washing. For low-stringency hybridisation, washing is carried out at 65°C with a buffer containing 2XSSC, 0.1 % SDS for 20 minutes. For medium-stringency, washing is continued at 65°C with a buffer containing 1XSSC, 0.1 % SDS for 2x 20 minutes, and for high-stringency filters are washed a further 2x 20 minutes at 65°C in a buffer containing 0.2XSSC, 0.1 % SDS. Probe labelling by random priming is performed essentially according to Feinberg and Vogelstein (1983) *Anal. Biochem.* 132(1), 6-13 and Feinberg and Vogelstein (1984) Addendum, *Anal. Biochem.*, 137(1), 266-267.

Substantially identical: refers to two nucleic acid or polypeptide sequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the sequence comparison algorithms given herein, or by manual alignment and visual inspection. This definition also refers to the complement of the test sequence with respect to its substantial identity to a reference sequence. A comparison window refers to any one of the number of contiguous positions in a sequence (being anything from between about 20 to about 600, most commonly about 100 to about 150) which may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Optimal alignment can be achieved using computerized implementations of alignment algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis. USA) or BLAST analyses available on the site: (www.ncbi.nlm.nih.gov). Furthermore, substantially

identical nucleic acid or polypeptide sequences perform substantially the same function.

Transcription factor: any protein required to initiate or regulate transcription of a gene, which may bind directly or indirectly to the DNA sequence of *cis*-elements of the gene (for example a WRKY transcription factor).

Transgene: refers to a polynucleotide sequence, for example a "chimeric gene", which is integrated into the genome of a plant by means other that a sexual cross, commonly referred to as transformation, to give a transgenic plant.

Transgenic plant: a plant harbouring a transgene stably integrated into host DNA and inherited by its progeny.

UTR: untranslated region of an mRNA or cDNA sequence.

Wild type: a plant gene, genotype, or phenotype predominating in the wild population or in the germplasm used as standard laboratory stock.

III. Isolation of a MAP Kinase Substrate 1 protein and its homologues

The present invention concerns the protein MAP kinase substrate 1 (MKS1), isolated from *Arabidopsis thaliana*, and homologous or orthologous plant MKS1 proteins. As described more fully below in the examples, MKS1 is a positive regulator of the SAR signal transduction pathway, and plays a key role in the regulation of SA levels and PR gene expression in response to pathogen attack. MKS1 was identified by its interaction with MPK4, first detected in a yeast 2-hybrid screen. MPK4 is a negative regulator of SAR that represses SA-mediated defence responses (Petersen *et al.*, 2000, *supra*). MKS1, isolated from *Arabidopsis thaliana*, is a polypeptide of 222 amino acids residues (Seq. ID No: 2; GI:18401970; At3g18690), having 11 putative phosphorylation sites. Interaction between MPK4 and MKS1 is further demonstrated to occur *in vitro*, and *in vivo* in *Arabidopsis* plants. Interaction between MPK4 and MKS1 can furthermore lead to phosphorylation of MKS1 at one or more phosphorylation sites, where

phosphorylation of residue S30 has been confirmed. MKS1, expressed as a GFP-fusion protein, is co-localised in the nucleus of leaf mesophyll cells, together with MPK4. The targeting of MKS1, as well as MPK4, to the nucleus is consistent with its role in the SAR signal transduction pathway and induction of PR gene transcription. MKS1, isolated from *Arabidopsis thaliana*, is encoded by the intron-less gene (Seq ID No: 1; GI:18401969; At3g18690), whose function was previously unknown.

MKS1 is shown to interact with down-stream components of the SAR signal transduction pathway, which are involved in the regulation of PR gene expression. The transcription factors WRKY25 (Acc.No:GI:15991726) and WRKY33 (Acc.No:GI:21105639) are identified as interaction partners of MKS1 by 2-hybrid screening and directed 2-hybrid assay. These transcription factors are Group 1 members of a large family of WRKY plant transcription factors, which are characterised by a N-terminal WRKY domain having the conserved amino acid sequence WRKYGQK, together with a zinc finger motif (Eulgem *et al.* 2000, *Trends in Plant Sci* 5: 199-206). WRKY proteins bind to highly conserved *cis*-acting W box elements (T)(T)TGAC(C/T), which are present in defence response genes, including PR1. Although the evidence for a role of WRKY transcription factors in regulating plant defence responses is convincing, the function of the majority of members of the WRKY family is yet to be elucidated. The phosphorylation of MKS1 by MPK4, combined with the protein-protein interaction between MPK4 and MKS1 and between MKS1 and WRKY25 and 33, clearly establish MKS1 as a key regulatory protein in the SAR signal transduction pathway.

SAR is a plant defence mechanism, which is widely conserved in the plant kingdom (Durner J. *et al.*, 1997 *Trends in Plant Science* 2: 266-274). Thus MKS1 homologues, which function as regulator proteins in the SAR signal transduction pathway, may be found in other plants, including crop plants. MKS1 homologues and orthologues can be identified by a standard protein-

protein BLAST or tblastn search against the database www: ncbi.nlm.nih.gov/blast/BLAST.cgi . Since the isolated *Arabidopsis* MKS1 is encoded by the *MKS1* gene sequence At3g18690 (GI:18401969), an nblastn search may similarly be performed to identify plant genes encoding MKS1 homologues and orthologues. The application of this approach is illustrated in the Examples, where MKS1 homologues or orthologues are identified in *Arabidopsis* (Seq ID No: 6; At1g21326), *Brassica oleracea* (Seq ID No: 10 and 14), *Glycine max* (Seq ID No: 16), and *Oryza sativa* (rice) (Seq ID No: 20), encoded by *MKS1* gene homologues or orthologues in *Arabidopsis* (Seq.ID.No:5; GI:22329704), *Brassica oleracea* (Seq ID No: 9, GI:17796488, BoBH544707; Seq ID No: 13, BoBOHBT92TR + BOGQI24TF), *Glycine max* (Seq ID No: 15; GI:8283399, GmBE020960), and *Oryza sativa* (rice) (Seq ID.No:19, OsCAD40925;), respectively. Additional MKS1 homologues or orthologues are found in rice (Seq ID No: 26, OsAP004654) maize (*Zea mays*) (Seq ID No: 27, ZmCC613160; Seq ID No: 28, ZmCC635639), tobacco (*Nicotiana tabacum*) and clover (*Medicago truncatula*) as exampled in figure 1B. In an alternative approach, nucleotide sequences encoding plant MKS1 homologues or orthologues can be identified in libraries constructed from plant genomic or cDNA by hybridisation screening with a polynucleotide probe comprising 20 or more consecutive nucleotides of an *MKS1* gene (for example At3g18690). Hybridisation screening is performed according to standard protocols, under conditions defined above. Plant genomic or cDNA may also be screened for nucleotide sequences encoding plant MKS1 homologues or orthologues by PCR, using primer sequences comprising 15 or more consecutive nucleotides of an *MKS1* gene (for example At3g18690), and a standard PCR amplification protocol as defined above. The PCR amplification of nucleotide sequences encoding MKS1 can also be performed using degenerate primers whose design is based on conserved amino acid sequences in MKS1, which can be identified by ClustalW alignment of MKS1 homologues or orthologues, as shown in the Examples. In the case that a MKS1 cDNA sequence is a partial sequence,

the corresponding full-length MKS1 cDNA may be generated using 5′ and 3′ RACE as defined above.

A MKS1 protein homologue or orthologue is characterised by a primary sequence that comprises domain 1 with sequence: IXGPRPXXLXVXXDSHXIKK and domain 2 with sequence: PVIIYXXSPKVIHTXXXEFMXLVQRLTG, wherein X refers to any amino acid residue, and conservatively modified variants thereof. A MKS1 protein homologue or orthologue is substantially identical to a MKS1 protein with Seq ID No: 2, 6, 10, 14, 16, 20, 26, 27 or 28, furthermore comprising amino acid sequence domains 1 and 2, (given above) or conservatively modified variants thereof. A nucleic acid molecule encoding a MKS1 protein homologue or orthologue is characterised by a nucleotide sequence that is a substantially identical to a nucleic acid molecule with Seq ID No: 1, 5, 9, 13, 15 or 19, or more preferably a conservatively modified variant thereof. Furthermore, a MKS1 protein homologue or orthologue is characterised by the properties of being a positive regulator of SAR, enhancing plant disease resistance, being phosphorylated by a MAP kinase and interacting with a transcription factor regulating SAR gene expression, e.g. WRKY transcription factor. Phosphorylation of MKS1 by a MAP kinase can be detected by *in vitro* phosphorylation assay as illustrated in the Examples. Interaction of MKS1 with a transcription factor can be detected by yeast 2-hybrid screens and directed 2-hybrid assays as illustrated in the Examples.

IV Transgenic plants with modified expression of MKS1 protein

A nucleic acid molecule encoding MKS1 protein can be used to modify and enhance MKS1 protein expression in a transgenic plant of the invention and thereby induce a SAR response and increase the pathogen resistance in the plant. The method provided by the invention can be utilised to induce SAR and confer disease resistance in a wide variety of plants. The coding sequence of an *MKS1* gene can be amplified by PCR using sequence

specific primers, for example: *Arabidopsis MKS1* (Seq ID No: 1) is amplified by the primer pair (Seq ID No: 3 & 4); *Arabidopsis MKS1* (Seq ID No: 5) is amplified by the primer pair (Seq ID No: 7 & 8); *Brassica oleracea MKS1* (Seq ID No: 9) is amplified by the primer pair (Seq ID No: 11 & 12); *Oryza MKS1* (Seq ID No: 19) is amplified by primer pair (Seq ID No: 21 & 22). *Glycine max MKS1* (Seq ID No: 15) comprises coding sequence for a part of MKS1 protein, and the complete MKS1 coding sequence may be generated by 5' and 3'RACE, as described above using primers for 3' extension (Seq ID.No: 17) and 5' extension (Seq ID No: 18). An expression cassette is constructed comprising a nucleic acid sequence encoding a MKS1 polypeptide, substantially identical to protein SEQ ID No: 2, 6, 10, 14, 16 or 20 and furthermore comprising a domain 1 (IXGPRPXXLXVXXDSHXIKK) and domain 2 (PVIIYXXSPKVIHTXXXEFMXLVQRLTG) amino acid sequence or conservatively modified variants thereof, wherein said nucleic acid sequence is operably linked to a heterologous or homologous promoter and 3' terminator. The expression casette can be transformed into a selected host plant using a number of known methods for plant transformation. By way of example, the expression cassette can be cloned between the T-DNA borders of a binary vector, and integrated into an *Agrobacterium tumerfaciens* host by transformation, and used to infect and transform a host plant (Hinchee *et al* 1988 *Bio/Technol.* 6:915-922, Ishida *et al.,* 1996 *Nat Biotechnol.* 14:745-50). The expression cassette is commonly integrated into the host plant in parallel with a selectable marker gene giving resistance to an herbicide or antibiotic, in order to select transformed plant tissue. Stable integration of the expression cassette into the host plant genome is mediated by the virulence functions of the *Agrobacterium* host. Binary vectors and *Agrobacterium tumefaciens*-based methods for the stable integration of expression cassettes into the majority of all dicotyledenous and monocotylenous crop plants are known, as described for example for rice (Hiei *et al.,* 1994, *The Plant J.* 6: 271-282) and maize (Yuji *et al.*, 1996, *Nature Biotechnology,* 14: 745-750). Alternative transformation methods,

based on direct transfer can also be employed to stably integrate expression cassettes into the genome of a host plant, as described by Miki *et al.*, 1993, "Procedure for introducing foreign DNA into plants", In: *Methods in Plant Molecular Biology and Biotechnology,* Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pp 67-88). Promoters to be used in the expression cassette of the invention include constitutive promoters, for example the 35S CaMV promoter (Acc.No:V00141, J02048), the *Arabidopsis* and maize *UBI1* gene promoter (Christensen *et al.*, 1992, *Plant Mol Biol* 18: 675-689), maize *ADH* gene promoter (Last *et al.* 1991 *Theor Appl Genetics* 81: 581-588), rice *ACT1* gene promoter (McElroy *et al.* 1990 *Plant Cell* 2: 163-172). In an alternative embodiment, an inducible promoter may be used in the expression cassette to direct MKS1 expression. Examples of suitable inducible promoters include the ecdysone agonist inducible promoter (Martinez *et al.* 1999 *Plant J.* 19: 97-106), glucocorticoid agonist inducible promoter (Aoyama and Chua, 1997 *Plant J.* 11: 605-612), copper inducible promoter (Mett *et al.* 1993 *Proc Natl Acad Sci USA* 90: 4567-4571), ethanol inducible promoter (Caddick *et al.* 1998 *Nature Biotech* 16: 177-180), tobacco *WUN1* promoter (Seibertz *et al.* 1989 *Plant Cell,* 1: 961-968) and the disease-inducible *WRKY28* promoter (gi:17064157; Dong *et al.*, 2003 *Plant Mol Biol.*, 51: 21-37). Additionally, the inducible *MKS1* gene promoter may itself be used to direct expression in the *MKS1* expression cassette. An example of a suitable tissue-specific promoter includes the promoter from the *Arabidopsis thaliana* RuBisCo small subunit gene NM_179480 [gi:30695946]. A terminator that may be used in the expression construct can for instance be the NOS terminator (Acc No: NC_003065) (SEQ ID No: 24), the terminator of the *Arabidopsis thaliana* RuBisCo small subunit gene NM_179480 [gi:30695946]. The recombinant vector comprising the MKS1 expression cassette is optionally transformed into a plant cell together with a selectable marker gene which is located on the same or a separate recombinant vector. Marker genes that facilitate selection of transformed plant cells, may encode peptides providing resistance to herbicide, antibiotic

or drug resistance, for example, resistance to protoporphyrinogen oxidase inhibitors, hygromycin, kanamycin, G418, gentamycin, lincomycin, methotrexate, glyphosate, phosphinothricin. Optionally, host plants transformed with an expression cassette encoding a MSK1 protein, can be crossed with a second non-transgenic plant and progeny expressing said MKS1 protein can then be selected and used in the invention.

Transgenic plants comprising a transgene expressing a MKS1 polypeptide can be used in a breeding program, in order select plants with enhanced agricultural performance that have inherited the transgene. Transgenic plants as well as plant progeny selected in such a breeding program may be cultivated for the purpose of harvesting a crop, where the crop may be vegetative plant parts, e.g. leaf or tuber, or reproductive parts including seed, caryopsis, cob or fruit.

V Plant disease resistance of transgenic plants with modified MKS1 expression

The expression of MKS1 in transgenic plants, transformed with a MKS1 expression cassette, will be determined by the promoter to which the MKS1 coding sequence is operably linked. Where MKS1 expression is placed under the control of a constitutive promoter, MKS1 will be expressed throughout the plant at all developmental stages. The expression pattern of MKS1 will in turn determine the SAR response pattern in the plant and the level of resistance to plant pathogen attack. Since MKS1 induces SA synthesis, all basal pathogen resistance mechanisms induced by SA will be up-regulated by MKS1 expression. Since MKS1 does not regulate expression of jasmonate-induced genes, its expression in transgenic plants will not impair jasmonate-dependent wound responses in a plant. Furthermore, since MKS1 appears to act upstream of NPR1 in the SAR signal transduction pathway, it is expected to regulate a broader range of disease responses in a plant. Methods for

assessing plant pathogen resistance are well known (Jach *et al.* 1995 *Plant J.* 8: 97-109 ; Whalen *et al.* 1991 *Plant Cell* 3: 49-60), and may be adapted according to the principal pathogens of the transgenic plant species. One method for assessing the resistance of a transgenic *Arabidopsis* plant, transformed with a MKS1 expression cassette, to a bacterial pathogen (*Pseudomonas syringae*) attack is given in the Examples. Other methods for evaluating disease resistance in plants are described by Crute *et al* 1994, Arabidopsis, Cold Spring Harbor Press, pp 705-747. Other examples of plant pathogens include the bacterial pathogens, *Erwinia* (for example *E. carotovora*), *Xanthomonas* (for example *X. campestris* and *X. oryzae*). Examples of fungal or fungal-like disease causing pathogens include *Alternaria, Ascochyta, Botrytis, Cercospora, Colletotrichum, Diplodia, Erysiphe, Fusarium, Gaeumanomyces, Helminthosporium, Macrophomina, Nectria, Perenospora parasitica, Phoma, Phymatotrichum, Phytophora, Plasmopara, Podosphaera, Puccinia, Puthium,Pyrenophora, Pyricularia, Pythium, Rhizoctonia, Scerotium, Sclerotinia, Septoria, Thielaviopsis, Uncinula, Venturia and Verticillium.*

The level of SAR in the transgenic plant can also be assessed by measuring the level of SA in the transgenic plant leaves, and the level of PR gene induction. Steady-state levels of PR mRNA can be quantitated by RNA blot hybridisation or alternatively by real-time PCR, as defined above. Application of these methods to the detection and quantitation of SAR in transgenic plants expressing MKS1 constitutively is illustrated in the Examples.

VI Isolated MKS1 and specific MKS1 antibodies

A nucleic acid molecule encoding the MKS1 protein can be operably linked to a promoter sequence to form a chimeric gene capable of directing expression of the MKS1 protein in a host cell. The nucleic acid molecule encoding MKS1 protein (ORF) can be fused in frame with a nucleic acid sequence encoding a tag. The expression of MKS1 as a fusion protein comprising a tag (e.g. 6x

histidine tag, or a glutathione-S-transferase tag) facilitates the purification of the expressed MKS1 protein. Affinity purification of tagged protein is well known in the art, and its application to the purification of MKS1 protein is described in the Examples. The chimeric gene can be cloned, as an expression cassette, in a recombinant vector, and transformed into a host cell. The expression cassette can be transformed into a bacterial cell e.g. *E.coli* and expression of tagged MKS1 protein can be controlled by an inducible promoter system, e.g. IPTG inducible promoters. Alternatively, an expression cassette can be transformed into a host plant cell, and transformed plants comprising the expression cassette can be selected. Protein extracts, prepared from tissue of the transformed plant expressing tagged-MKS1 protein, can be used for the affinity purification of tagged-MKS1.

Tagged-MKS1, MKS1, or peptide fragments thereof, can be used for the production of specific polyclonal and monoclonal antibodies. Synthetic peptides having amino acid sequence identity to 10 or more consecutive amino acid residues of a MKS1 protein can be synthesised and used as antigen for the production of specific MKS1 antibodies. It is common to couple the synthetic peptide to a carrier protein, e.g. PPD (Purified Protein Derivative; Bardarov *et al.* 1990, *FEMS Microbiology Letters* 71: 89-94), to enhance the stability of the antigen and improve the presentation of the antigen to the immune humoral response system. Polyclonal and monoclonal antibodies can be raised, screened and tested according to standard protocols, as given by Harlow and Lane (1988) *Antibodies, A Laboratory Manual,* Cold Spring Harbour Publ. NY. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive for a protein. For example, solid-phase ELISA immunoassays, immunoblots, or immunohistochemistry are regularly used for this purpose. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

Examples

MPK4 is a plant protein kinase whose regulatory functions include default repression of SA-dependent SAR, a pathway that primarily mediates resistance to certain biotrophic pathogens via PR gene expression. In addition, MPK4 is involved in the activation of PDF1.2 expression in response to jasmonate and ethylene, pathways that mediate resistance against necrotrophs and wounding herbivores (Petersen et al., 2000 *supra*). Since the regulatory functions of MPK4 are dependent on its kinase activity, it is likely that MPK4 interacts with and phosphorylates protein substrates which directly or indirectly lead to the control of gene expression appropriate to various pathogen responses. Hence, the identification and isolation of a protein, which interacts with and is phosphorylated by MPK4, would provide a key regulator of SA-dependent SAR in plants.

Example 1
Arabidopsis MAP Kinase Substrate 1 (MKS1) interacts with MPK4

A yeast two-hybrid screen was employed to identify proteins that interact with the MPK4 protein. The yeast two-hybrid screen, first described by Fields and Song in 1989 (*Nature* 340: 245-24) is a common method used to detect protein-protein interactions. This screen exploits inherent properties of transcription factors, namely that are composed of two separate domains: a DNA-binding domain and a transcription activation domain. A physical association of the two domains of a transcription factor is required in order for it to bind to a promoter and activate transcription of a downstream gene. DNA sequences encoding fusion proteins, comprising the DNA-binding domain or the activation domain of a transcription factor, can be constructed and co-expressed in yeast. Interaction between the two fusion proteins will result in a functional transcription factor. If a DNA binding domain-MPK4 fusion protein

(bait), and an activation domain fused to an MSK1 interacting protein (prey) are simultaneously expressed in yeast, a functional interaction between the two fusion proteins can be detected by the transcription of nutritionally essential genes and reporter genes cloned in yeast. The yeast two-hybrid screen is commonly based on the detection of yeast colonies in which transcription of these essential genes enables cell growth on histidine- or leucine-deficient media, and detectable β-galactosidase activity.

An *Arabidopsis* cDNA library fused to the activation domain of a transcription factor (prey) was screened for potential MPK4 interacting partners using the following yeast two-hybrid system. *Saccharomyces cerevisiae* strain PJ69-4A (MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ; (James *et al.*, 1996, *Genetics* 144: 1425-1436) was used as host strain for two hybrid screening. Cells were grown at 30°C in liquid YPD medium (www: clontech.com) or on YPD agar plates. Transformed yeast cells were grown in liquid SD medium or on SD agar (Minimal SD Agar Base; www:clontech.com) plates supplemented with drop-out supplements (www:clontech.com) lacking specific amino acids. Yeast cells were transformed using the lithium acetate/polyethylene glycol method (Ito *et al.*, 1983, *J Bacteriol* 153: 163-168). Library screening was performed with the MPK4 bait encoded by the full-length *MPK4* cDNA from *Arabidopsis thaliana* Ecotype L*er*, cloned into the *Bam* H1 site in pGBD-C1 (James *et al* 1996, *supra*). Both GAL4-based library screens were performed with the *Arabidopsis* MATCHMAKER cDNA library cloned in pGAD10 GenBank #U13188 (www: clontech.com/techinfo/manuals). Two independent screens of the library were conducted with the MPK4 bait, and in total the number of screened clones (6×10^7) covered the library 20 times. Subsequently 7.4 million colonies were screened with MKS1as bait, corresponding to 25 times the number of individual clones in the library.

A single full-length cDNA, designated MAP Kinase Substrate 1 (MKS1), corresponding to the intron-less *Arabidopsis* gene At3g18690, was found to interact with MPK4 in the yeast two-hybrid screen, shown in Figure 1A. A similar interaction was observed after switching MPK4 and MKS1 as prey and bait, respectively. To test the specificity of the MAP kinase interaction, the interaction of MKS1 with other plant MAP kinases was tested in the yeast two-hybrid assay. The following MAP kinase cDNA sequences were cloned as AD fusions in pGAD424 (www: clontech.com): MPK3 (nucleotides 149-1261 of NM_114433) using *Bam*HI/*Sal*1 sites; MPK5 (nucleotides 466-1218 in NM_117204) using *NcoI*/*Not*1 sites; MPK6 (nucleotides 116-1303 in NM_129941) using *Bam*HI/*Sal*1 sites; MPK17 (nucleotides 1-1740 in NM_126206) using *NcoI*/*Not*1 sites. In contrast to MPK4, the MPK3, 5, 6 or 17 (Ichimura *et al.* 2002, *Trends in Plant Sci.,* 7, 301-308) preys did not interact with the MKS1 bait (Figure 1A), confirming the specificity of the MKS1-MPK4 interaction.

MKS1 is a protein of 222 amino acid residues having a predicted molecular mass of 24 kDa, and the sequence of Seq ID No: 2 (At3g18690). MKS1 is encoded by nucleotides 80 to 748 of the Arabidopsis gene At3g18690; GI: 18401970 (SEQ ID No: 1). MKS1 contains 11 putative MAP kinase phosphorylation sites (Ser-Pro), indicated in Figure 1B, based on sequence homology to other described phosphorylation sites (minimal consensus sequence S/TP; Sharrocks *et al.*, 2000, *Trends in Biochem Sci.*, 25: 448-453). The coding sequence for MKS1 was used in a standard protein-protein BLAST and tblastn search against the database at the www:ncbi.nlm.nih.gov/blast/BLAST.cgi and www:arabidopsis.org/Blast sites. The BLAST searches identified the following nucleic acid sequences comprising ORFs coding for previously unknown proteins, now identified as: *Arabidopsis MKS1* gene homologue (Seq ID No: 5; Acc.No:At3g21326) encodingMKS1 protein homologue (Seq ID No: 6), *Brassica oleracea MKS1* gene homologue (Seq ID No: 9; Acc.No:BH544707; GI:17796488) encoding

MKS1 protein homologue (Seq ID No: 10), *Brassica oleracea* (Seq ID No: 13; Acc. No:BOHBT92TR + BOGQI24TF) encoding MKS1 protein homologue (Seq ID No: 14), *Glycine max MKS1* gene homologue (Seq ID No: 15; Acc.No: BE020960) encoding MKS1 protein homologue (Seq ID No: 16), rice *MKS1* gene homologue (Seq ID No: 19; Acc.No:CAD40925; GI: 21740554) encoding MKS1 protein homologue (Seq ID No: 20), rice *MKS1* gene homologue (Acc.No: OsAP004654) encoding MKS1 protein homologue (Seq ID No: 26), maize *MKS1* gene homologue (Acc.No: ZmCC613160) encoding MKS1 protein homologue (Seq ID No: 27), maize *MKS1* gene homologue (Acc.No: ZmCC635639) encoding MKS1 protein homologue (Seq ID No: 28, which all share sequence identity with *Arabidopsis* MKS1 (Seq ID.No: 2) and comprise Domains 1 and 2, as shown in the protein alignment given in Figure 1B. The alignment was generated with the aid of CLUSTAL programs (clustalw.genome.ad.jp; Jeanmougin,F. *et al.*, (1998) *Trends Biochem Sci*, 23, 403-5; Thompson,J.D., *et al.* (1997) *Nucleic Acids Research*, 24:4876-4882; Higgins, D. G., *et al.* (1996) Using CLUSTAL for multiple sequence alignments. Methods Enzymol., 266, 383-402. 5) Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) *Nucleic Acids Research*, 22:4673-4680; Higgins,D.G., *et al.* (1992) *CABIOS* 8,189-191; Higgins,D.G. and Sharp,P.M. (1989) *CABIOS* 5,151-153; Higgins,D.G. and Sharp,P.M. (1988) *Gene* 73,237-244). Furthermore, MKS1 homologues or orthologues are also found in maize (*Zea mays*), tobacco (*Nicotiana tobacum)* and clover (*Medicago truncatula*) (Figure 1B). Arabidopsis MKS1 protein (At3g18690) shares a sequence identity of 84.8% with *Brassica oleracea MKS1* (Acc.No:BH544707) and 78.4% with *Brassica oleracea* (Acc no: BOHBT92TR + BOGQI24TF). The identified MKS1 homologues all comprise amino acid sequence domains 1 and 2, or conservatively modified variants thereof.

Example 2

Arabidopsis* MPK4 interacts with and phosphorylates MKS1 *in vitro

A. MPK4-MKS1 interaction *in vitro*

To substantiate the interaction between MPK4 and MKS1, detected in the yeast two-hybrid screen, *in vitro* interaction assays (pull-down assays) were performed with recombinant MPK4 and MKS1 proteins. Recombinant MKS1 was obtained by bacterial expression according to the following procedure. The full-length MKS1 coding sequence (At3g18690 nucleotides 80 to 748) was cloned in-frame with the glutathione-S-transferase (GST) gene in the *Xho* I site of pGEX-5X plasmid (www: amershambiosciences.com). Expression of the recombinant protein in *E. coli* BL21 (pLysS) cells (www:novagen.com) was induced with 0.1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) at 30°C for 3–4 h, and 2% ethanol was added before induction. GST protein was similarly expressed in *E.coli* from the pGEX-5X plasmid. GST and GST-fusion proteins were purified from whole cell extracts of *E. coli* by binding to glutathione–Sepharose 4B beads (www: amershambiosciences.com), in the presence of proteinase inhibitors (2 µg/ml leupeptin, 1 mM AEBSF (4-(2-Aminoethyl)-bezenesulfonylfluoride.HCl), 2 µg/ml antipain, 5 mM EDTA, 5 mM EGTA, 2 µg/ml aprotinin). Proteins used in pull-down assays were not eluted from the glutathione sepharose beads. ^{35}S-methionine-labelled MPK4 was generated by coupled transcription-translation of the bait plasmid pGBKT7-MPK4 from the two hybrid screen, using a T7 coupled reticulocyte lysate system (www: promega.com/tbs/tb126/tb126.pdf).

Pull-down assays were preformed as follows: 10 µl ^{35}S-MPK4 was mixed with 200 µl 1% BSA in Bead Binding (BB) Buffer (BB Buffer; 50 mM KPO_4 pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 2µg/ml leupeptin, 1 mM AEBSF, 2 µg/ml antipain, 2 µg/ml aprotinin), incubated on ice for 15 min, and then centrifuged for 10 min at 4°C. The supematant was added to 2-5 µg GST or GST-fusion protein bound to sepharose beads in 200 µl 1% BSA in BB Buffer and incubated for 2 hrs at 4°C with rotation. The beads were washed 3 times with 1 ml wash buffer (50 mM KPO_4, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10%

glycerol, 5% Triton X-100) with proteinase inhibitors and were then subjected to SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis; Laemmli, 1970 *Nature* 227: 680) separation on 15% gels.

The pull-down assay demonstrated that *in vitro* synthesized MPK4 (Fig. 2A, lane 1) interacts with and was bound by recombinant MKS1-GST (Lane 3), but not by GST alone (Lane 2), thereby confirming the MPK4-MSK1 interaction detected in the yeast two-hybrid screen.

B. MPK4 phosphorylation of MKS1 *in vitro*

The ability of MPK4 to phosphorylate putative MAP kinase Ser-Pro phosphorylation sites in MKS1 was investigated by *in vitro* phosphorylation assays. Full-length and C-terminally truncated histidine-tagged MSK1 were expressed and purified from *E. coli*. MKS1 nucleotide sequence (nucleotides 80 to 748 of At3g18690) encoding full-length MKS1 protein, was cloned into the *Xho* I site of the pET15b plasmid (www:novagen.com). Nucleotide sequences encoding MKS-1 with terminal deletions, C1-C3, were constructed by restiction digest of the MKS1-HIS containing pET15b vector. The C1 deletion was generated with *BstB*I and *Bpu*1102I, the C2 deletion with *Nhe*I and *Bpu*1102I, and the C3 deletion with *Sty*I and *Bpu*1102. The digested plasmids were end-filled by incubation with 3 U Klenow enzyme and 10 μM deoxyribonucleotides for 30 minutes at 37°C, and then re-ligated by overnight incubation with ligase enzyme at 16°C. The plasmids constructs encoding full-length MKS1 (amino acids 1-222), C1-MKS1 truncation (amino acids 1-196), C2-MKS1 truncation (amino acids 1-123) and C3-MKS1 truncation (amino acids 1-73), as shown in Figure 1B, were transformed into *E. coli* BL21 (pLysS) cells (www: novagen.com), expression was induced with 0.1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) at 30°C for 3–4 h, adding 2% ethanol prior to induction. Expressed MKS1 was extracted using BugBuster and Benzonase assisted protein extraction, and purified by affinity

binding of the histidine tag to Ni-NTA resin, according to the instructions of the manufacturer (www:novagen.com).

HA (influenza hemagglutin antigen)-tagged MPK4 (HA-tag is 6 X YPYDVPDYA) was expressed by transgenic *Arabidopsis* plants (Petersen *et al.*, 2000, *supra*). The HA-MPK4 was purified from protein extracts of the plants as described (Romeis *et al.*, 1999, *Plant Cell* 11: 273-287), except that a buffer change was not made prior to immunoprecipitation (Romeis *et al.*, 1999, *Plant Cell* 11: 273-287). 100 µg of total protein was immunoprecipitated from the plant protein extract with 2 µg/ml monoclonal 12CA5 HA-antibody (Boehringer) by affinity to the HA tag. Protein concentrations were determined with the Bradford dye-binding procedure (Bradford, 1976, *Anal Biochem* 131: 248-254). The resulting sepharose beads, with immunoprecipitated MPK4, were washed in kinase buffer (200 µM ATP, 80 mM Tris-HCl, pH 7,5, 8 mM EGTA, 120 mM $MgCl_2$, 4 mM Na_3VO_4, 4 mM DTT) to remove the immunoprecipitation buffer and suspended, as a 50% slurry, in kinase buffer.

Phosphorylation assays were performed by mixing 10 µl MPK4-sepharose slurry, 5 µg substrate protein and 0,4 µl 300 µM ^{32}P-γ-ATP (3 µCi) with kinase buffer in a final volume of 30 µl. The assay samples were incubated for 1 h with agitation at 30°C, where after the assay proteins were separated by SDS-PAGE, and the gels subsequently dried on Whatmann 3MM paper and the radiolabelled proteins detected on a phosphorimager screen.

HA-tagged MPK4, immunoprecipitated from *Arabidopsis* plants, is shown to *in vitro* phosphorylate MKS1 as efficiently as myelin basic protein (MBP; Sigmasource), which is a standard MAP kinase substrate, as shown in Figure 2B, lanes 1 versus 5). Immunoprecipitated extracts of non-transgenic *Arabidopsis* plants (wt) failed to phosphorylate MKS1 (Figure 2B, lanes 6-8) confirming that the HA-antibody specifically immunoprecipitates HA-tagged MPK4. Furthermore, a mutant HA-tagged MPK4, with substitutions in the

kinase activation loop abolishing MPK4 activity (T201A/Y203F; Petersen *et al.*, 2000, *supra*) was similarly found not to phosphorylate MKS1 or MBP.

In order to identify which sites in MKS1 are phosphorylated by MPK4, C-terminal MKS1 truncations (C1, C2, C3), lacking some of the putative Ser-Pro phosphorylation sites (Figure 1B), were tested in the phosphorylation assay. HA-tagged MPK4 readily phosphorylated both full-length and C-terminal MKS1 truncations, including C3 MKS1, which retains only 2 putative phosphorylation sites (Ser30 and Ser72), as seen in Figure 2B, lanes 2-4. In order to map the functional phosphorylation sites in the C3 MKS1 protein, the encoded MSK1 sequence was altered from Ser30 to Ala30 (S30A) by *in vitro* mutagenesis, by substituting the codon TCA for GCA in the full-length and C3 truncated *MKS1* gene. Although the mutant C3 truncated MKS1 (C3-S30A) was not phosphorylated by HA-tagged MPK4, the mutant full-length MKS1 (S30A) was phosphorylated (Figure 2C, lanes 1 and 2 versus 3 and 4). This indicates that MPK4 phosphorylates MKS1 at Ser30, as well as other additional sites in the MKS1 protein.

A synthetic 22 amino acid peptide (Pep22), corresponding to amino acid residues 13-35 of MKS1 and comprising Ser30, shown as in Figure 1B, was synthesized by KJ Ross (www:tagc.com). Pep22 is an efficient competitor of full-length MKS1 for phosphorylation by MPK4, when added to the *in vitro* assay in a molar ratio of 1:1 (Pep22:MKS1) as shown in Figure 2D, top. The Flg22 peptide, with amino acid sequence QRLSTGSRINSAKDDAAGLQIA, which is known to activate immediate pathogen responses via the flagellin receptor, involving MPK3, 5, 6 and 17 as well as WRKT 22 and 29 (Asai *et al.* 2002, *Nature* 415: 977-983), was used as a control in this assay. Since the Flg 22 peptide did not compete MKS1 phosphorylation (Figure 2D, bottom) it is likely that the Pep22 domain of MKS1 specifically interacts with MPK4.

Example 3

Antibodies for MKS1 detection

To provide tools for the detection of MKS1 expression *in vitro* or *in vivo* in single or multicellular organisms, polyclonal (pa-Pep22) and monoclonal antibodies (ma-Pep22 & ma-Pep22p) were raised against the peptide Pep22 (SDQQNQKRQLQICGPRPSPLSVH), corresponding to amino acid residues 13-35 of MKS1. Ten to twelve-week old female Balb/cCF1 F1-hybrid mice were used to raise both polyclonal and monoclonal antibodies. The mice were primed with 0.2 mL live BCG vaccine, delivered intraperitoneally. One month later the mice were immunised with the antigen Pep22 coupled to PPD (Purified Protein Derivative; Bardarov *et al.* 1990, *FEMS Microbiology Letters* 71: 89-94), absorbed onto the adjuvant $Al(OH)_3$. The total volume of vaccine per immunisation was 500 µL, containing 15 µg of PPD and 1 mg of adjuvant. The antigen was injected intraperitoneally at 2-week intervals. To prepare polyclonal antibodies from the immunised mice, blood samples were collected 10 days after each immunisation and assayed for specific recognition of HIS-tagged MKS1 protein, expressed and purified from *E. coli*, followed by SDS-PAGE separation and semi-dry transfer and immunoblotting (Current protocols, www:wiley.com). Western blots were developed using alkaline phosphatase conjugated anti-mouse antibody (Promega). Monoclonal antibodies were prepared from immunised mice found to produce positive antisera, essentially as described by Kohler and Milstein (1975) in *Nature* 256: 495-497, as modified by Reading (1982) in *J Immunol Methods* 53: 261-291. After hybridoma cell fusions, culture supernatants were tested for specific recognition of HIS-tagged MKS1 protein, by enzyme-linked immunoabsorbent assay (ELISA; Current protocols, (www: wiley.com) and immunoblotting as described above for polyclonal antibodies.

Polyclonal antibody, pa-Pep22, specifically recognised MKS1 present in extracts of *E. coli* and wild type *Arabidopsis* plants, as shown by Western blotting in Figure 3A. The same result was obtained using the monoclonal antibody ma-Pep22 (not shown). Monoclonal antibody ma-Pep22 (HYB 330-01), specifically recognised and immunoprecipitated MKS1 present in extracts of wild type *Arabidopsis* plants, since the immunoprecipated MKS1 was detected by the pa-Pep22 polyclonal antibody, as seen in lane 1 (upper band) of a Western blot (Figure 3B). The lower band is due to binding of the secondary anti-IgG antibody to the ma-Pep22 light chain, which was also present in a control immunoprecipitation with ma-Pep22 where plant extract is omitted, seen in lane 2 (Figure 3B).

Example 4

Arabidopsis* MPK4 interacts with MKS1 *in vivo

Interaction between MKS1 and MPK4 *in vivo* in Arabidopsis plants was demonstrated by the ability of the MKS1 specific monoclonal antibodies to co-immunoprecipitate MPK4 with MKS1 from leaf extracts. Leaf protein extracts were prepared as described in Example 2B, from transgenic *mpk4* plants complemented to wild type by a functional HA-tagged MPK4 gene (Fig. 3C; Petersen *et al.*, 2000, *supra*). Immunoprecipitates of the leaf extracts were analysed by SDS-PAGE and Western blots, which were probed with anti-HA antibody to detect HA-tagged MPK4. As shown in Figure 3C lane 1, ma-Pep22 monoclonal antibody co-immunoprecipitated HA-tagged MPK4, which was detected with the anti-HA antibody. The co-immunoprecipitated HA-tagged MPK4 co-migrated with MPK4 immuno-detected in whole plant extracts (lane 1 versus lane 3). Monoclonal antibody (ma-Con), that does not detect MKS1 in plant extracts, was unable to immunoprecipitate MPK4 (lane 2). The upper bands immunodetected in lanes 1, 2 and 4 of Figure 3C are likely due to binding of the secondary anti-IgG to the heavy chain of the immunoprecipitating monoclonal IgGs. Only the

upper band was detected in a mock-plant extract containing MaPep22 (lane 4).

Example 5

Transgenic *Arabidopsis* plants with modified MKS1 expression

Transgenic plants, expressing elevated or reduced levels of MKS1 protein, were generated in *Arabidopsis thaliana* ecotype Landsberg erecta (Ler) via *Agrobacterium*-mediated transformation, according to the floral dip method (Clough and Bent, 1998 *Plant J.*, 16:735-43). Transgenes were inserted between the T-DNA borders of pCAMBIA binary vectors, comprising the NPTII (kanamycin) resistance gene, and then transformed into *Agrobacterium*, and stably integrated into the *Arabidopsis* genome.

Constitutive over-expression of MKS1 in plants was obtained by the stable integration of CaMV 35S-MKS1 transgenes in *Arabidopsis*. The *Arabidopsis* MKS1 coding sequence (nucleotides 80-748 of Seq ID. No: 1 (At3g18690)) was amplified from its respective gene by PCR using a 5' primer (Seq ID.No: 3) and 3' primer (Seq ID No: 4). A transgene comprising the CaMV 35S promoter sequence (GI: 2173396; with Seq ID. No: 23), operably linked to a MKS1 coding sequence (nucleotides 80-748 of At3g18690), was generated by replacing the GUS ORF in pCAMBIA1301 (AF234297) by the MKS1 sequence, ligated with *Nco I/Bst* EII linkers. *Arabidopsis* transformants were selected by resistance to the antibiotic hygromycin incorporated into the seedling growth medium. Transformants with an integrated copy of the CaMV 35S-MKS1 transgene in the *Arabidopsis* genome were identified by northern blotting with a MKS1 probe (nucleotides 80-748 of At3g18690) and western blotting with maPep22.

Self-fertilisation of *Arabidopsis* transformants, with an integrated copy of the CaMV 35S-MKS1 transgene, led to seed formation, with the stable

inheritance of the transgene in the progeny, and subsequent generations. Cross-pollination of the primary transformed plants or their progeny with control, non-transformed plants, generated progeny that inherited the transgene according to Mendelian genetics.

Silencing of MKS1 expression in plants by RNA interference (Chuang and Meyerowitz, 2000, *Proc Natl Acad Sci. U S A.* 97: 4985-4990) was obtained by the stable integration of a CaMV 35S-MKS1 RNAi transgene. The MKS1 coding sequence (nucleotides 80-748 of Seq ID No:1 (At3g18690)) was first inserted in the plasmid SLJ1382B1 (Andrea Ludwig and Jonathan DG Jones, Sainsbury Laboratory, UK), derived from plasmid SLJ4D4 (Jones *et al.* 1992, *Transgenic Research* 1: 285-297). The MKS1 coding sequence was cloned, in opposite orientations, on either side of an intervening intron in pSLJ1382B1, as 5' *Xba* I/*Fse* I- and 3' *Asc* I/*Xho*-linkered fragments. The intron had the sequence: GTAAGTTTCTGCTTCTACCTTTGATATATATATAATAATTATCATTAATTAG TAGTAATATAATATTTCAAATATTTTTTTCAAAATAAAAGAATGTAGTATAT AGCAATTGCTTTTCTGTAGTTTATAAGTGTGTATATTTTAATTTATAACTTT TCTAATATATGACCAAAATTTGTTGATGTGCAG and Seq ID. No: 25. The resultant RNAi cassette was excised with *Eco* RI and *Hind* III and cloned into corresponding sites in pCAMBIA3300 (derivative of pCAMBIA1201, AF234293). This construct was transformed into ecotype Col-0, and transformants were selected by resistance to spraying with the herbicide BASTA (Glufosinate-ammonium; www: bayercropscience.com).

Transgenic plants with elevated levels of MKS1, expressed under control of the constitutive promoter CaMV 35S (35S-MKS1), were identified by immunodetection of MKS1 in plant protein extracts analysed by western blotting with the polyclonal antibody pa-Pep22, a shown in Figure 4A. Transgenic plants in which MKS1 expression was silenced by RNA interference were similarly identified by immunodetection of MKS1 levels in

plant protein extracts (Figure 4A). The 35S-MKS1transgenic plants exhibited semi-dwarfism in contrast to the dwarf habit of *mpk4* mutants (Figure 4B). The MKS1-RNAi plants were phenotypically wild type in their growth habit (not shown).

Example 6

Properties of transgenic *Arabidopsis* plants with modified MKS1 expression

A. MKS1 regulates the expression of pathogen resistance genes, but not wound and methyl jasmonate response genes, in plants.

The steady-state levels of MKS1, PR and wound-induced gene transcripts were measured in total RNA samples extracted from *Arabidopsis* plants which were analysed by Northern blotting and hybridization with DNA probes according to standard protocols. DNA probes were amplified by PCR, with sequence-specific primers, from the following cDNA or genomic DNA templates: MKS1 (nucleotides 80-748 of At3g18690), PR1 (nucleotides 84-530 in M90508), *PDF1.2* (EST 37F10T7), *VSP* (nucleotides 3-236 in ATTS0751/GBGA288), WR3 (WR3 probe in AtT5G50200, described by Leon J. *et al.*, 1998, *Mol Gen Gen* 258: 412-419).

35S-MKS1 transgenic plants accumulated elevated levels of MKS1 mRNA compared to wild-type, consistent with increased MKS1 synthesis in these plants (Figure 5A). Levels of the pathogen resistance PR1 mRNA, were enhanced in 35S-MKS1 transgenic plants and in *mpk4* mutant plants when compared to wild-type plants (Figure 5A, lane1 versus 2 and 3).

MKS1, in contrast to MPK4, is shown not to be involved in the response to wounding and necrotrophic attack in plants. Plants respond to wounding and necrotrophic attack by the transcriptional activation of jasmonate and/or ethylene responsive genes including *VSP*, *WR3* and *PDF1.2*, in which MPK4

is known to play a regulatory role (Petersen *et al.*, 2000, *supra*; Andreasson E. and Mundy J, unpublished). The steady-state levels of these wound-induced genes was determined in *Arabidopsis* plants, subjected to wounding by making 1 to 3 cuts over the mid vein with a pair of scissors. *VSP* and *WR3* mRNAs were induced in wild-type plants within 2 hours of wounding (Figure 5B, lane 7), but were undetectable or greatly reduced in *mpk4/ NahG* plants expressing the bacterial salicylate hydroxylase that degrades SA (Figure 5B, lane 3). The same results were also seen following wounding of the *mpk4* mutant (not shown). Silencing MKS1 expression in RNAi-MKS1 plants did not prevent a wild-type wounding response with the accumulation *VSP* mRNA (Figure 5C, lanes 2 versus 4). These results indicate that MKS1 is not required for wound-responsive *VSP* expression. Silencing or over-expression of MKS1 did not significantly affect the levels of PDF2.1 mRNA accumulation following 48hr of MeJA treatment (Fig. 5D). This indicates that MKS1, in contrast to MPK4 (Petersen *et al.* 2000 *supra*) is not required for MeJA responsive PDF1.2 expression.

B. Salicylic acid levels are enhanced in 35S-MKS1 transgenic plants

The steady-state free and glycosylated salicylic acid content of *Arabidopsis* plants was analysed in plant extracts prepared by grinding plant tissue in liquid nitrogen, extracting the ground tissue in methanol, following by an ethylacetate:cyclopentane:isopropanol partition of the extract according to (Newman *et al.*, 2001, *Mol Plant-Microbe Interactions* 14: 785-792). The salicylic acid content was analysed by HPLC using a diode array detector between 180-350nm, as previously described Newman *et al.*, 2001, *supra*. Salicylic acid levels were significantly elevated in 35S-MKS1 transgenic plants in comparison to wild-type plants, as shown in Figure 6A.

C. Pathogen resistance is enhanced in 35S-MKS1 transgenic plants

Resistance to the plant pathogen *Pseudomonas syringae* is shown to be controlled by MKS1 expression levels in transgenic plants. Four-week-old

Arabidopsis plants were infiltrated with a suspension of $1x10^5$cfu/ml of virulent *Pseudomonas syringae* pv. *tomato* DC 3000 strain. Bacterial growth on infected plants was subsequently assayed by grinding four 0.5 cm^2 leaf pieces in 10mM $MgCl_2$ for each sample. Dilutions were distributed on NYG *agar* plates containing rifampicin, cycloheximin and kanamycin, and colonies were counted, as previously described (Parker *et al.* 1996, *Plant Cell* 8: 2033-2046). 35S-MKS1 transgenic plants exhibited increased resistance to *P. syringae* DC3000, as seen for *mpk4* plants, as shown in Figure 6B. The disease response of 35S-MKS1 transgenic lines expressing different levels of MKS1, indicated that MKS1 expression is directly correlated with PR1 expression and resistance to *Pseudomonas* attack (data not shown). In contrast, of MKS1-RNAi plants were significantly less resistant to *P. syringae* DC3000 than wild type plants (Figure 6C) confirming the key role of MKS1 in the development of SAR.

E. Localisation of MKS1 expressed in transgenic plants

Green fluorescent protein (GFP) expressed in plant cells can be detected by virtue of its fluorescent properties, and GFP-protein fusions have provided a valuable tool for determining the whole plant and subcellular expression pattern of proteins of interest (Stewart, 2001, *Plant Cell Rep.* 20:376-82). *Arabidopsis* plants transformed with MKS1-GFP gene fusions, under control of CaMV 35S or MKS1 promoters, were generated to determine MKS1 cellular localisation. The MKS1 coding sequence with *Eco* RI linkers (nucleotides 80 to 748 in At3g18690) was N-terminally fused in frame with a GFP coding sequence, operably linked to a CaMV 35S promoter in the binary vector pCAMBIA 1302 (AF234297). The MKS1-GFP gene fusion cloned in pCAMBIA 1302 was placed under the control of the MKS1 promoter by substituting the CaMV 35S promoter by a 1.9 kb MKS1 promoter fragment (complement of nt 15531-13589 of BAC MVE11) having *Nco I*/*Bst* EII linkers. The MPK4-GFP fusion was made by cloning a *Not*I linkered genomic fragment including 1150 bp promoter region from Ler genomic DNA cloned

into pAVA393 (Arnim *et al.*, 1998 *Gene* 221: 35-45). The control 35S-GUS-GFP fusion was included in the pCAMBIA1302, a derivative pCAMBIA1303. The transgenes in the binary vectors were transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation and transgenic lines were selected as described in Example 5.

GFP expressed in mesophyll cells of young leaves of the transformed lines was visualised by confocal microscopy. GFP fluorescence was detected with a Zeiss LSM 510 laser-scanning microscope applying the 488 nm line of the argon laser and the corresponding dichroic mirror and a 505-530 nm band-pass filter. The generated images of GFP fluorescence in cells are vertical projections of variable numbers of optical sections.

The phenotype of the transgenic lines expressing the MKS1-GFP fusion protein was similar to that of the 35S-MKS1 transgenic lines expressing enhanced MKS1 levels. This indicates that MKS1 retains functional activity when expressed as a GFP-MKS1 fusion protein. Similarly, the MPK4-GFP fusion protein is functional and correctly targeted when expressed in transgenic plants, since it is able to complement the *mpk4* mutant to wild type (Brodersen, Mattsson and Mundy, unpublished data). The GUS-GFP fusion protein, which lacks any specific subcellular, or extracellular targeting signals, was primarily localized to the cytoplasm of 35S GUS-GFP transgenic plants, as shown in Figure 6D. GFP-MKS1, as well as GFP-MPK4, were localised in the nucleus of mesophyl cells, consistent with the demonstrated *in vivo* interaction of these two proteins and their transcriptional repression of downstream SAR effector genes under normal growth conditions (Figure 6D).

In conclusion, transgenic plants with elevated levels of MKS1 expression show increased salicylic acid (SA) levels, PR gene expression and pathogen resistance, demonstrating that MKS1 is a key component of the SAR signal transduction pathway in plants controlling SAR and plant pathogen

resistance. During negative regulation of SAR by MPK4 in wild-type plants, MKS1 is presumably phosphorylated, at one or more sites.

Example 7

***Arabidopsis* MKS1 interacts with WRKY 25 and 33 transcription factors**

MKS1 is shown to be a key component of the SAR signal transduction pathway in plants, whose overexpression enhances SA levels and PR gene expression. The regulatory role of MKS1 is likely to be mediated by interaction with additional down-stream members of the pathway, including transcription factors. A yeast two-hybrid screen, with MKS1-BD as the bait, was used to identify proteins capable of interaction with MKS1. The MKS1-BD fusion was constructed by inserting the full-length MKS1 coding sequence (nucleotides 80-748 of At3g18690) into the *Nco* I restriction site of pGBKT7, and transformed into *S. cerevisiae* strain PJ69-4A (www: clontech.com). A GAL-4 based library screen in yeast of Arabidopsis MATCHMAKER cDNA libraries was performed as described in Example 1. 7. 4 million colonies were screened with the MKS1 bait, corresponding to 25 times the number of individual clones in the library. Two MKS1 interactors, the transcription factors WRKY25 (GI:15991725) and WRKY33 (GI:21105638), were identified in this screen, as shown in Figure 1A. WRKY 33 and 25 are among the 70, or more, WRKY transcription factors predicted in *Arabidopsis*, which show amino acid sequence similarity and both belong to the group I WRKYs (Eulgem *et al* 2000 *supra*). Five different truncated WRKY33 proteins interacted with MKS1 in the yeast library, the shortest corresponding to the C-terminal 188 amino acids of WRKY33. This region comprises the C-terminal WRKY domain and a region denoted the A-motif (Eulgem *et al.*, 2000, *Trends in Plant Sci* 5: 199-206).

The specificity of MKS1 interaction with WRKY transcription factors was examined in directed yeast two-hybrid assays with WRKY26 and WRKY29. Full-length cDNA WRKY26 (AF224699, nucleotides 23-949) and WRKY29

(AF442394, nucleotides 1-915) was fused with the nucleotide sequence encoding an AD domain in pGADT7 (www: clontech.com) using *Bam* H1 sites. WRKY26, of unknown function, is the next closest homolog to WRKY25 and 33, while the less similar WRKY29 positively regulates innate immunity responses involving MPK3 and 6 (Asai *et al.* 2002, *Nature* 415: 977-983). However, neither WRKY26 nor WRKY29 interacted with MKS1 in this assay (Figure 1A), indicating that the interaction of MKS1with WRKY25 and 33 is specific. No activity of the reporter His3, Ade2 or lacZ gene products was detected when any fusion protein construct was co-transformed with the corresponding empty vectors (data not shown).

MKS1 is shown to be a positive regulator in the SAR signal transduction pathway, interacting with the MAP kinase MPK4 and the transcription factors WRKY25 and WRKY33. The defence response pathways, triggered by pathogen attack or wounding, which involve a series of signalling steps controlled by regulator proteins leading to the expression of resistance genes, are outlined in a model presented in Figure 7. It is proposed that the negative regulator MPK4 represses SAR by phosphorylating and interacting with MKS1 to form a complex. The MPK4-MKS1 complex may, in turn, phosphorylate the transcription factors WRKY25 and 33 that may repress transcription of a salicylic acid promoter factor. The interaction of WRKY factors with promoters (W box motifs) is known to be phosphorylation dependent (Eulgem *et al.* 2000, *supra*).

MKS1 is a key regulatory protein of plant SAR and thereby controls the ability of plants to survive pathogen attack. Transgenic plants expressing enhanced levels of MKS1 protein show a significantly increased level of disease resistance. Thus transgenic plants comprising a transgene expressing enhanced levels of MKS1 may, by virtue of their increased disease resistance, produce a crop with a larger yield. Furthermore, the crop yield of these transgenic plants will be less dependent on the application of

fungicides and bactericides, which are expensive and often have a negative environmental impact. The SAR response is common to many members of the plant kingdom and hence the use of MKS1 proteins to up-regulate the pathogen defence response in a wide range of plants lies within the scope of the present invention.

Claims

1. A transgenic plant having increased expression of a positive regulator of systemic acquired resistance (SAR) and enhanced disease resistance characterised by a transgene comprising a nucleic acid sequence encoding a MAP kinase substrate 1 (MKS1) polypeptide.

2. The transgenic plant of claim 1, wherein said MKS1 polypeptide has a primary amino acid sequence comprising a domain 1 with sequence: IXGPRPXXLXVXXDSHXIKK and a domain 2 with sequence: PVIIYXXSPKVIHTXXXEFMXLVQRLTG, or conservatively modified variants thereof, wherein X refers to any amino acid residue.

3. The transgenic plant of claim 1, wherein said MKS1 polypeptide comprises an amino acid sequence selected from the group consisting of: SEQ ID No. 2, 6, 10, 14, 16, 20, 26, 27, 28 and conservatively modified variants thereof.

4. The transgenic plant of claim 3, wherein said MKS1 polypeptide is encoded by a nucleic acid sequence selected from the group consisting of: SEQ ID No. 1, 5, 9, 13, 15, 19, and conservatively modified variants thereof.

5. The transgenic plant of claims 1, 2 or 3, wherein said transgene comprises a homologous promoter.

6. The transgenic plant of claim 1, 2 or 3, wherein said transgene is a chimeric gene comprising a heterologous promoter.

7. The transgenic plant of claim 6, wherein said heterologous promoter is selected from the group consisting of: constitutive promoter, tissue specific promoter, and inducible promoter.

8. The transgenic plant of claim 1, 2 or 3, wherein said plant is a dicotyledonous plant.

9. The transgenic plant of claim 1, 2 or 3, wherein said plant is a monocotyledonous plant.

10. The transgenic plant of claim 8, which is selected from the group consisting of: potato, tomato, tobacco, carrot, radish, sweet potato, turnip, canola, sunflower, soybean, sugarbeet, bean, pea, chicory, lettuce, broccoli, cabbage, cauliflower, spinach, asparagus, onion, garlic, eggplant, pepper, celery, squash, pumpkin, melon, cucumber, apple, pear, plum, peach, cherry, quince, apricot, nectarine, orange, strawberry, raspberry, blackberry, pineapple, banana, avocado, papaya, mango and sugar cane.

11. The transgenic plant of claim 9, which is selected from the group consisting of: barley, maize, oats, rice, rye, sorghum and wheat.

12. Seed from the transgenic plant of claim 8 or 9.

13. A method for producing the transgenic plant of claim 1, 2 or 3, characterised by introducing an expression cassette comprising said transgene encoding said MKS1 polypeptide into a plant and selecting the transgenic plant and its progeny expressing said MKS1 polypeptide.

14. The method of claim 13, wherein the expression cassette is introduced into the plant through transformation.

15. The method of claim 13, wherein the expression cassette is introduced into the plant by sexual crossing with a transformed plant comprising a MKS1 transgene.

16. A recombinant vector comprising the transgene of claim 1, 2 or 3.

17. A method for detecting increased expression of MKS1 polypeptide in the transgenic plant of claim 1, 2 or 3, characterised in reacting an anti-MKS1 antibody with a protein extract derived from said plant.

18. The anti-MKS1 antibody of claim 17.

19. The anti-MKS1 antibody of claim 18, comprising a polyclonal antibody.

20. The anti-MKS1 antibody of claim 18, comprising a monoclonal antibody.

21. Use of the transgenic plant according to any one of claims 1, 2, 3 and 12 for the cultivation of a crop.

22. The crop of claim 21.

23. Use of the transgenic plant according to any one of claims 1, 2, 3 and 12 in a breeding program.

24. A plant selected in the breeding program of claim 23 having said transgene comprising a nucleic acid sequence encoding a MKS1 polypeptide.

Abstract

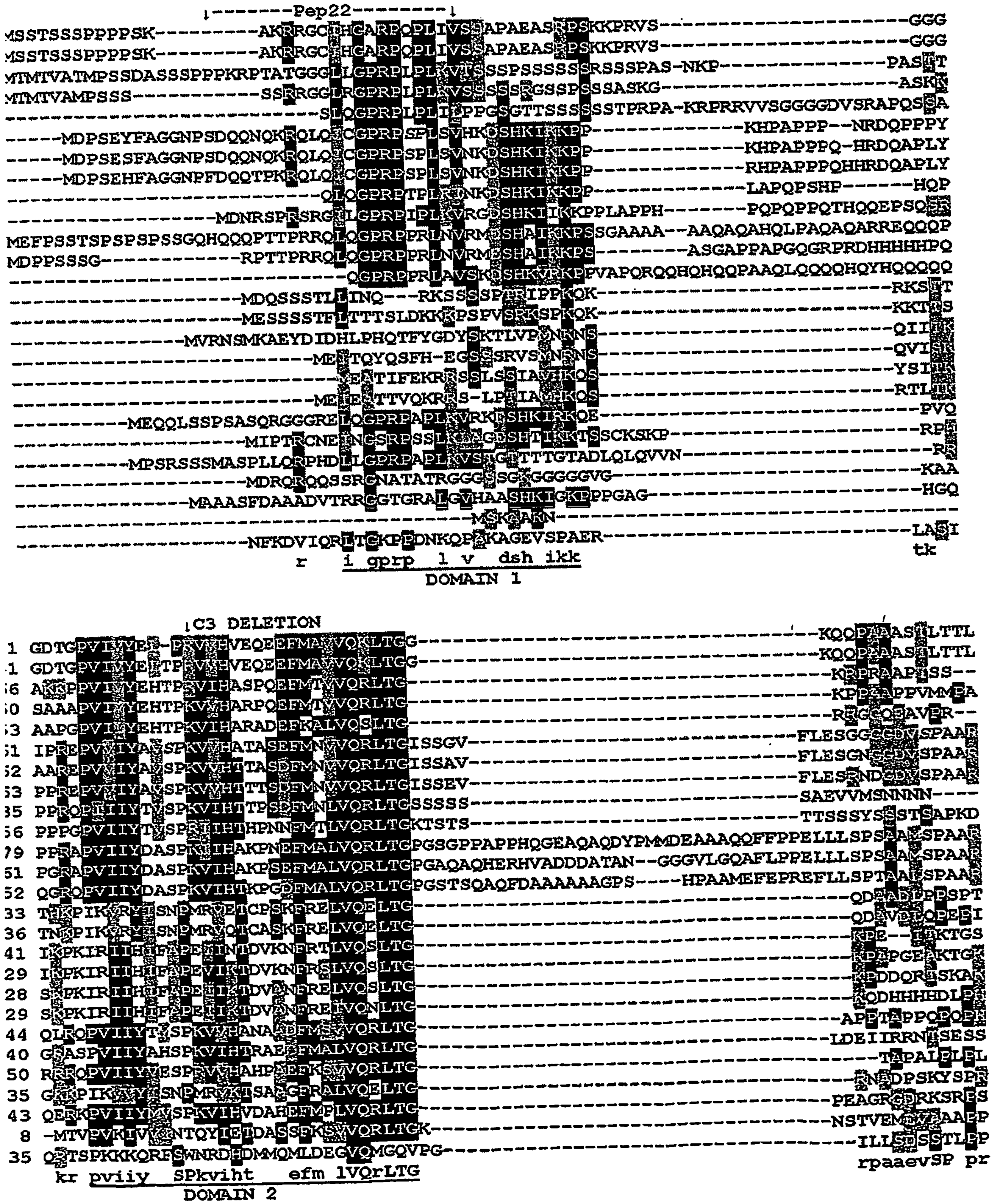
Plant disease resistance and SAR regulator protein

The invention provides a transgenic plant having increased expression of a positive regulator protein of systemic acquired resistance (SAR), thereby enhancing the SAR response and pathogen resistance of the plant. The positive regulator protein is a component of a signal transduction pathway leading to (SAR), and is a MAP kinase protein (MPK4) substrate, and interacts with transcription factors.

Figure 1 A.

BD fusion	AD fusion	-His growth	-Leu growth	β-gal activity	-Ade-Trp growth
MPK4	MKS1	+	+	+	+
MKS1	*MPK4*	+	+	+	+
MKS1	*MPK3*	-	-	-	+
MKS1	*MPK5*	-	-	-	+
MKS1	*MPK6*	-	-	-	+
MKS1	*MPK17*	-	-	-	+
MKS1	WRKY25	+	+	+	+
MKS1	WRKY33	+	+	+	+
MKS1	*WRKY26*	-	-	-	+
MKS1	*WRKY29*	-	-	-	+
MPK4	*WRKY25*	-	-	-	+
MPK4	*WRKY33*	-	-	-	+

gure 1B (1)



Patent- og
Varemærkestyrelsen

2 8 NOV. 2003

Modtaget

igure 1B (2)

```
                    ↓C2 DELETION
96  PAADQVAGGDHAAAAAAAADPLVLAG-GQRQPAPAPAIDGDHPAAPPHSPPADA--------FLLSPSSFFLSPTAMHALQ
97  PAADQVAGGDHAAAAAAAADPLVLAGGQQRQPAPAPAIDGDHPAAPPHSPPADA--------FLLSPSSFFLSPTAMHALQ
108 --LPSGTVSPAATAEEGCGDPLLLATGGRQQ-AAAPAPPTATALLPSSAAAAAG--------MLLSPG-FLFSPSTSQAIQ
94  YDAPAMVEQGAAACGAGGDPLLLAAGGRQA-AASAPAQPPAAPSP---AMAAG--------MLLSPG-FVFSPNAVQSIQ
95  ---------PPASSQQTAGGDLLVLAAGHHQH-VPPPPP----LPSP---GGAAG--------FLLSPGSFLFSPAAVQAIQ
113 LASTENASPRGGKE-------PAARDEAVEINTAAEEAAEFGGYAPGILSPSPAA--------LPTASTGTFSPMYHQGGM
114 LAATENASPRGGKEPV----MAAKDEAVEIAATAAEEAAELSGYAPGILSPSPAA--------LPTASAGIFSQMATHQGG
115 LAATENASPRGGKE--------------PVESSTAAEEAAEFGCYVPGILSPSPAA--------LPAVPAGIFSPMFHLGGM
83  ---------------------------TTHVDPFNNGGGGAVSPAARYATAEKAA---------SPMGKKHAVLLPSVNNIIS
108 ASTAVDTSAGLISPAARFAVTEKANASNELSTFVGGEGTADQYYHYHHHHHHQE---------QQHQNQGFERPSFHHAGT
59  LATAERSVRPMP------EPAPEYVDATNGGGGGGVDDGGLAAALGSIRPGILSP---LPSSLPPAAAVPGQFSPLPFDASC
39  LATAERSVRPVP------APAPAPDYAADG-------------HPRGAARPREAPR---HPVPAAVLAAAGRRVGPVAAAAA
28  LAAAERSVRPLPPHHAPAAVPPYFGAANDDAFFAPGSADADSASAALGPPASRPGILSPAALPPAASTGAFSPMPFDPSC
76  TF------TAVDLHRPCESEMNLEP-------LDGEVRGEYYSPADEEVFNAP-QA----------SAGLSGFFSSG------
79  YS------PSSDDHNLSPPAENLAPRALHQEPFGERDSDCYEPANAEDMFAPDQA----------SAGFSGFFSNG------
82  KK------KITNTNFPAPPGLPQDSGQEHTAELDNRIIG--SHAVKEEWGS-------------SSNTNTYFDFDGSIDLD
72  KR------AGSRIATPQEPVCDDHQPANRLSGFTGLLANGGNHQVKEEWGSGDQS----------TSNTNTYFDLEGSIQDG
71  PR--RDMHSLHRQVQDMINTEKLREPEHCDQEFCLNAEELSMTWNGNNGAGESS----------GGFLNGGGDFEGFIQEG
72  QKGYKRNPASRRSHDHHEVHDANKSHGFCINSDEEEEAMASMTWNGN--GDESS----------GGFLNGGGDLDGFIQEG
88  HPTALAQMPPQPSFPFHLQQQDAWPQQGHSPAATEQAAARSSGADLPPLPSILSP--VPGTVLPAIPASFFSPPSLISPG
85  SSVATEEVNVGDDNTAAPFSQDRTQRQKLTDMPAFTPSSAALFGSPTQLAYMSP------------NRADSFRPLVFKSE-
91  KFPFHFHFPSSVGAASRALLPLTSSTSSGVMSVAATVADTTCGGGSPVQQAPNHH-------MLASSDPCDAPPPPSLCRVE
78  ASADDDDGGGGGGGELAAASDGAGEPGPGAAAASPDTGAAAASDAADALVAAG----------HPAAATFDDEGGGGG
88  TGGAGGAGEAEPSEGARNKTRAAPPARVKARALNRPAVSAATTRRQQAAADASP----------PSGLMLFQDLSPARGAA
53  PTAAANYYRHGYANWDDQSSRALISETESIARKPSPSLGRGMSFNDFDNAFKEAP----------PVDDLLRLYADEYRSNY
80  ISSENFSLVSLPSTPLYTFSPETFSPATSYIFSPEKNMLAVPSNIDRKGTIPTAQS----EVYPQMNMHQARILSKFLGG
     aam      h aat   a aa pl   tltq   a am   aa lt  la g    m aal          lpsa  sgifSP  tm  a  m

                          ↓C1 DELETION
168 EEEAARRVVEATSTGLGVQSGKSSQDTVNLHADANAAAAAALAARHSSRGPRSSTQPP------------------------ a
                                                                                   ----- b
170 ELAALF------------------------------------------------------------------------------ c
177 ELNPLF------------------------------------------------------------------------------ d
162 ELSPLF------------------------------------------------------------------------------ e
152 EIIS-------------------------------------------------------------------------------- f
179 FS-------PAIPLGLFSPAGFMSPFFRSPGFT-------------SLVASPTFADFFSHIWDQD------------------- g
182 MF-------SPG---LFSPAGLMSPFG---FA--------------SLVASPTFADLFSHIWG--------------------- h
175 FS-------PALPPGLFSPAGLMSPGYAS------------------LASPNFADFFSHIWDP--------------------- i
129 DV-------E-------------------------------------------------------------------------- j
180 LSPGPNSLPSVSPDFFSTIGPTDPQGFSSFFNDFNSILQSSPSKIQSPSSMDLFNNFFDS------------------------ k
231 ISWLNELSPILRAASAGAASSGSGGGGSGGNTSNGGAARPPPSYYADPFVPSPRHLLATPTVPSPATCAELFSNLPDL l
199 RPQQRQLAQRAQPHPPGSVHGQRSAPLAHAHGPTGGSRQP-------------------------------------------- m
208 LSWLSELSPFLPSAGTRAAAAGLLDQAPFAPSPRSSLLLSTPTMPS-PATFSVLEFFSSPNFPDL------------------- n
129 -----------------------------------FYNVNALGSIGSL------------------------------------- o
139 -----------------------------------FYNVNDFGSIDSM------------------------------------- p
142 -----------------------------------EDDNIFSSRWFDLQQQ---------------------------------- q
138 GEDYFSSFPMRSSSSSQVEGFIFNNNNNNNNNNNNNNNNTNFDTKAHNSS----------------------------------- r
140 G-------------------EFPYLPLSSIDASASSNSSS--------------------------------------------- s
141 G-------------------EFPYLPFTIDPAVASSSHLHGNVFAEPHHYA---------------------------------- t
166 PFLGATTTSSAAPSTSPSPMGGSAYYWDLFNMQQQQHYHHQN------------------------------------------ u
    ------------------------------------------------------------------------------------- v
165 SSNTTAVVSSSTNTSKNYE------------------------------------------------------------------ w
148 GYYDDDDDDIFRSQLLDTSYSVFSPPTLLYDHPHSKV------------------------------------------------ x
159 LKGEHLPLVSPAWLHHVGDHFLSPAGAAAALGSPSPSFVDDIFGTLSSQQQ---------------------------------- y
125 ------------------------------------------------------------------------------------- z
156 AKPP---------------------------------------------------------------------------------
```

a: OsBAC15955 b: Os8355.t00567 c: ZmCC661221 d: Os8360.t05160 e: ZmBM340911
f: MKS1 At3g18690 f: BoBH544707 g: BoBOHBT92TR + BOGQI24TF h: GmBE020960
i: At1g21326 j: OsCAD40925 k: ZmCC613160 l: ZmCC635639 m: At2g41180
n: AtAL390921 o: AtAL138658 p: At2g44340 q: AtT46022 r: At2g42140
s: OsAP004654 t: At1g68450 u: ZmCC442903 v: OsAP003260 w: ZmCC700850
x: Mtacre169 z: MtAC143340.1

Figure 2.

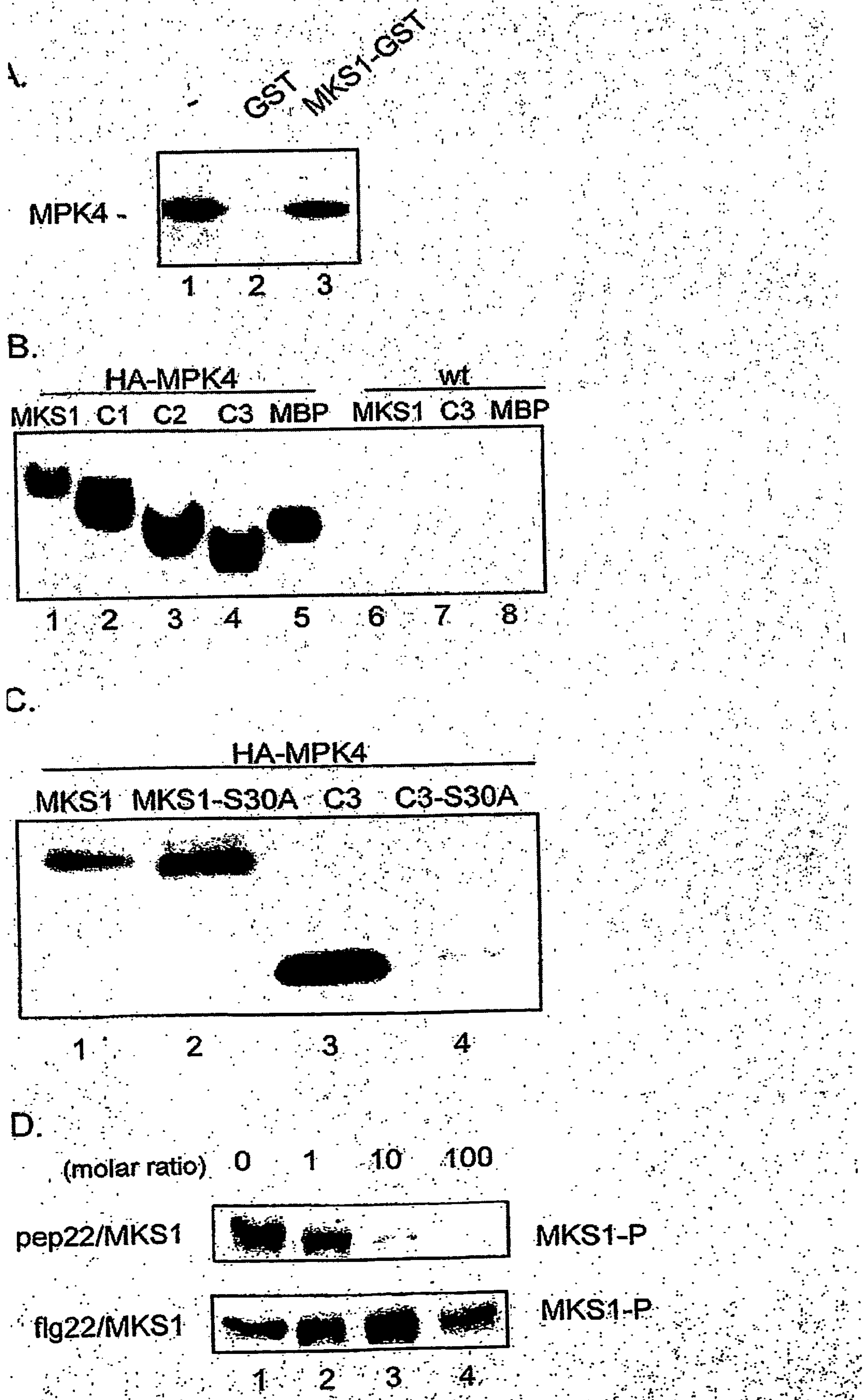

A.
-
GST
MKS1-GST
MPK4 -
1 2 3
B.
HA-MPK4
wt
MKS1 C1 C2 C3 MBP MKS1 C3 MBP
1 2 3 4 5 6 7 8
C.
HA-MPK4
MKS1 MKS1-S30A C3 C3-S30A
1 2 3 4
D.
(molar ratio) 0 1 10 100
pep22/MKS1
MKS1-P
flg22/MKS1
MKS1-P
1 2 3 4

Figure 3.

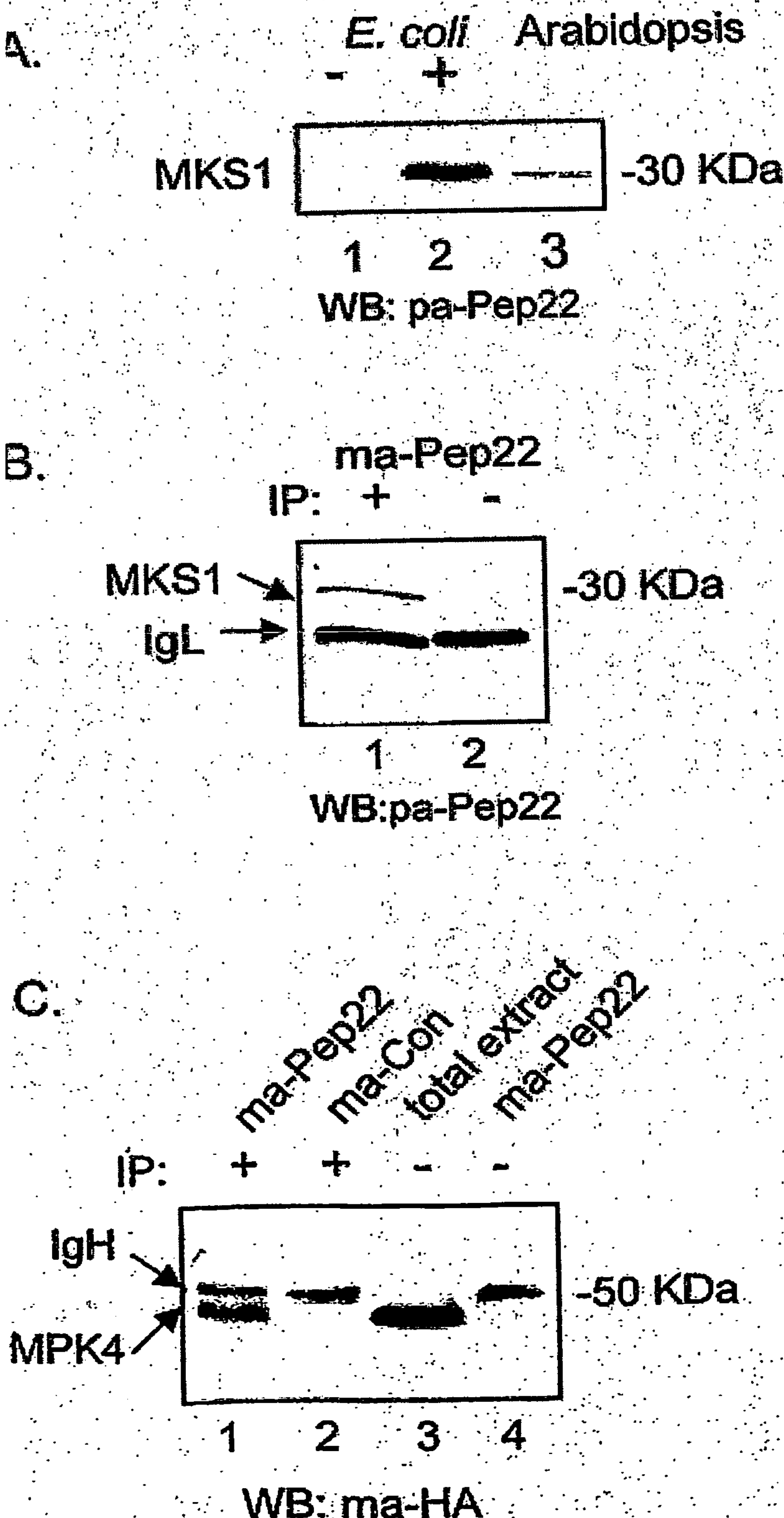
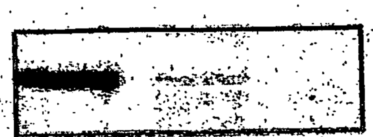

A.
E. coli
Arabidopsis
- +
MKS1
-30 KDa
1 2 3
WB: pa-Pep22
B.
ma-Pep22
IP: + -
MKS1
IgL
-30 KDa
1 2
WB:pa-Pep22
C.
ma-Pep22
ma-Con
total extract
ma-Pep22
IP: + + - -
IgH
MPK4
-50 KDa
1 2 3 4
WB: ma-HA

Figure 4.

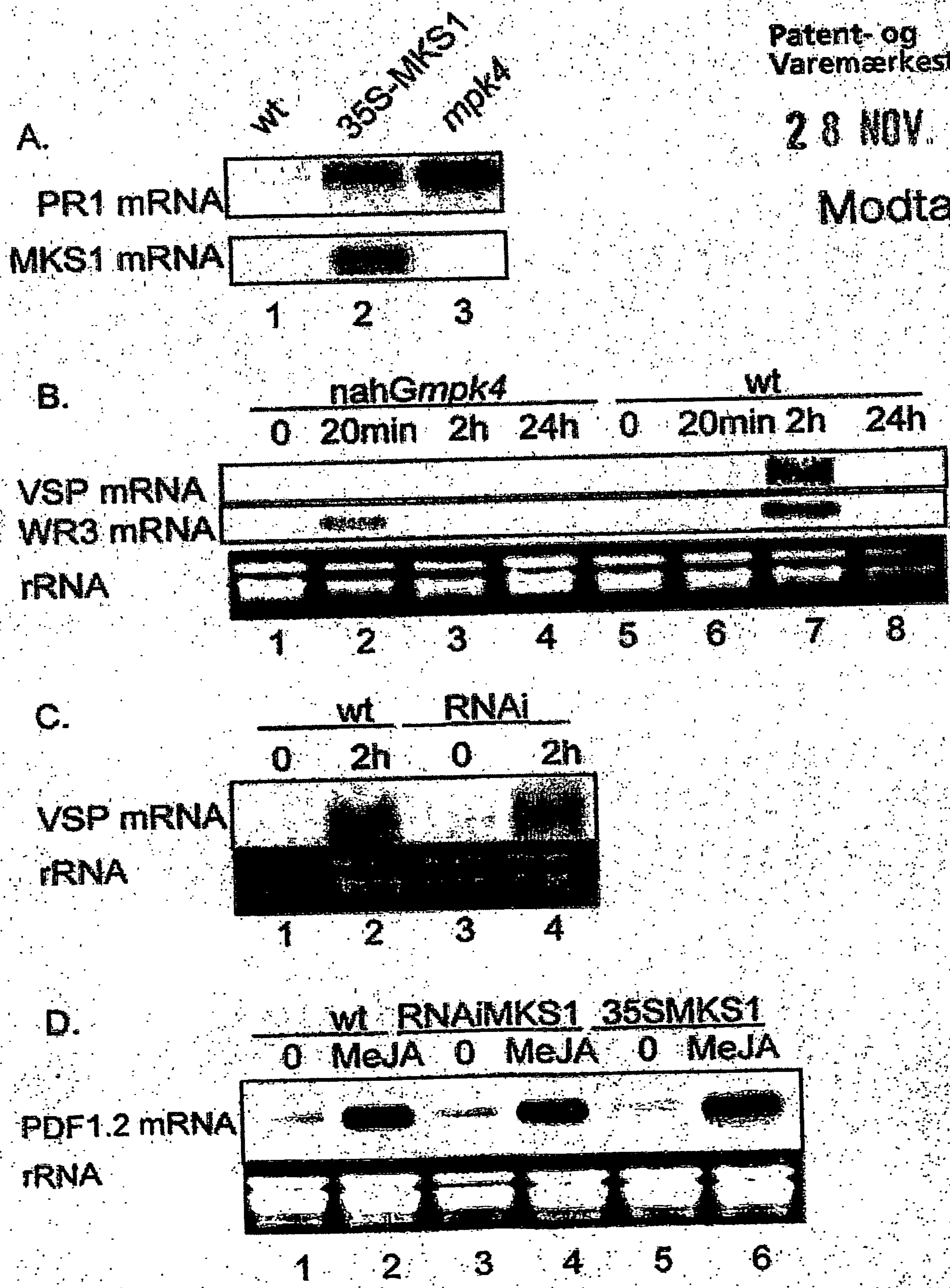
A.


35S-MKS1
wt
RNAi-MKS1
MKS1
-30 KDa
1
2
3
WB: pa-Pep22

B.


wt
35S-MKS1
mpk4

Figure 5.



A.
wt
35S-MKS1
mpk4
PR1 mRNA
MKS1 mRNA
1
2
3
B.
nahGmpk4
wt
0
20min
2h
24h
0
20min
2h
24h
VSP mRNA
WR3 mRNA
rRNA
1
2
3
4
5
6
7
8
C.
wt
RNAi
0
2h
0
2h
VSP mRNA
rRNA
1
2
3
4
D.
wt
RNAiMKS1
35SMKS1
0
MeJA
0
MeJA
0
MeJA
PDF1.2 mRNA
rRNA
1
2
3
4
5
6

Figure 6.

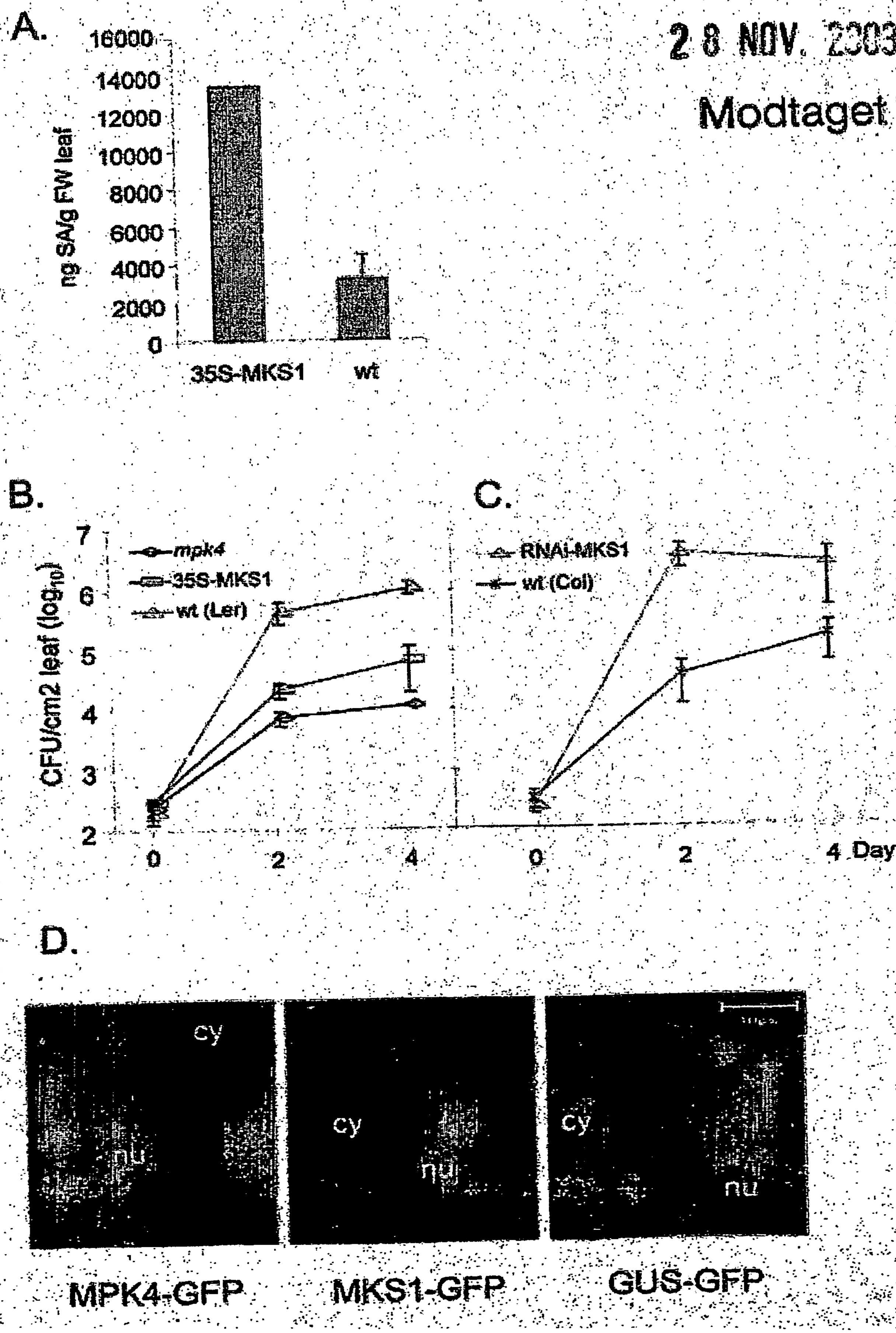
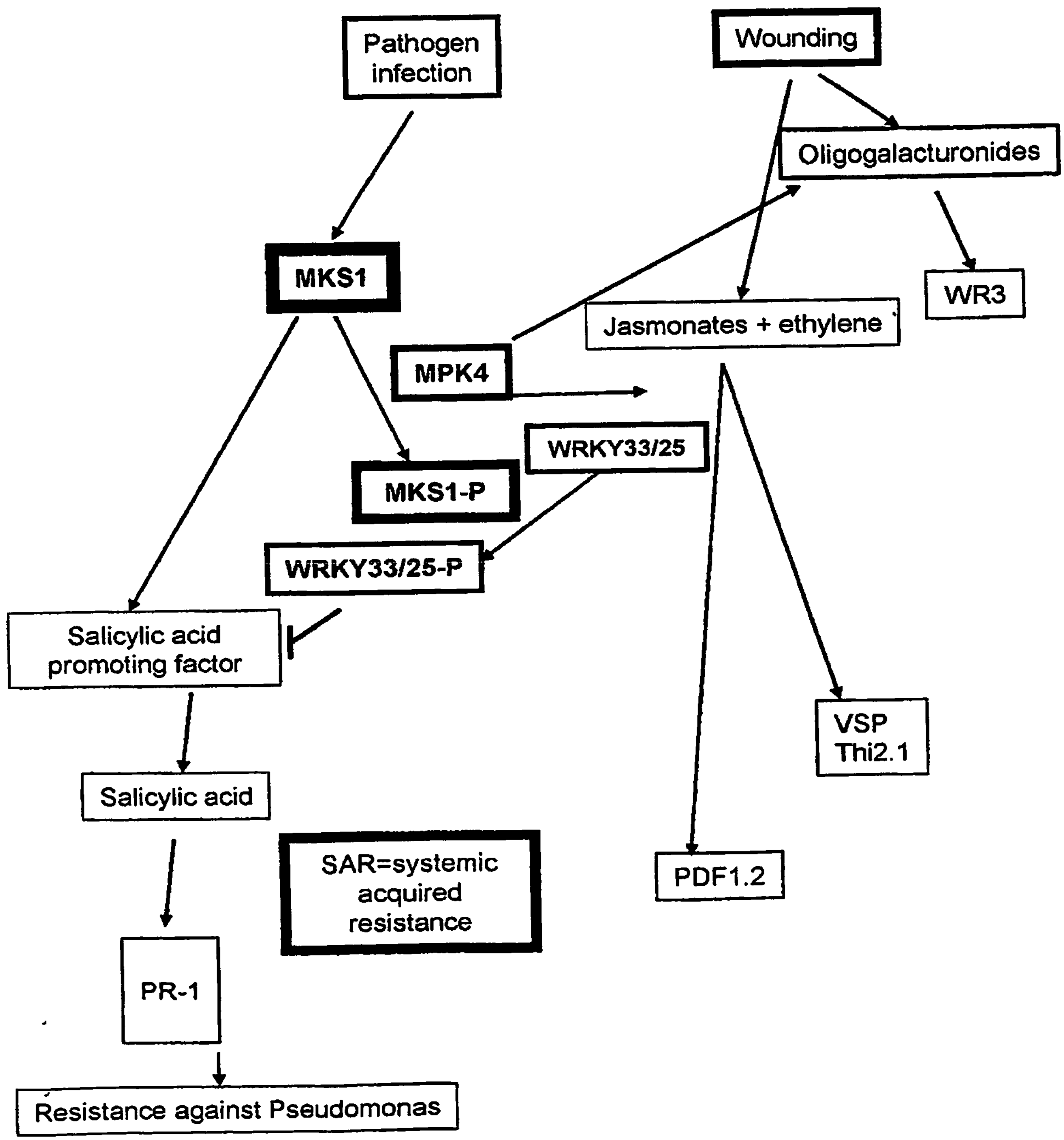

A.
16000
14000
12000
10000
8000
6000
4000
2000
0
ng SA/g FW leaf
35S-MKS1
wt
B.
7
6
5
4
3
2
CFU/cm2 leaf (log_{10})
mpk4
35S-MKS1
wt (Ler)
0
2
4
C.
RNAi-MKS1
wt (Col)
0
2
4 Day
D.
cy
nu
cy
nu
cy
nu
MPK4-GFP
MKS1-GFP
GUS-GFP

Figure 7.


Pathogen infection
Wounding
Oligogalacturonides
MKS1
WR3
Jasmonates + ethylene
MPK4
WRKY33/25
MKS1-P
WRKY33/25-P
Salicylic acid promoting factor
VSP
Thi2.1
Salicylic acid
SAR=systemic acquired resistance
PDF1.2
PR-1
Resistance against Pseudomonas

SEQUENCE LISTING

<110> Copenhagen University Tech Trans Enheden
        Mundy, John

<120> Plant disease resistance and SAR regulator protein

<130> P200301025

<160> 28

<170> PatentIn version 3.2

<210> 1
<211> 669
<212> DNA
<213> Arabidopsis sp.

<220>
<221> CDS
<222> (1)..(669)

<400> 1
```
atg gat ccg tcg gag tat ttt gcc ggc ggc aat cct tcc gat caa cag      48
Met Asp Pro Ser Glu Tyr Phe Ala Gly Gly Asn Pro Ser Asp Gln Gln
1               5                   10                  15

aac cag aag cgg cag ctt cag atc tgt ggt cct cgt cct tca cct ctt      96
Asn Gln Lys Arg Gln Leu Gln Ile Cys Gly Pro Arg Pro Ser Pro Leu
            20                  25                  30

agt gtt cac aaa gac tct cac aaa atc aag aaa cct cca aaa cac cct     144
Ser Val His Lys Asp Ser His Lys Ile Lys Lys Pro Pro Lys His Pro
        35                  40                  45

gcg ccg ccg cca aat cgt gac caa ccg ccg ccg tat att cct aga gag     192
Ala Pro Pro Pro Asn Arg Asp Gln Pro Pro Pro Tyr Ile Pro Arg Glu
    50                  55                  60

ccg gtg gtt atc tac gcc gta tcc ccc aag gtt gta cac gca acc gcg     240
Pro Val Val Ile Tyr Ala Val Ser Pro Lys Val Val His Ala Thr Ala
65                  70                  75                  80

tct gag ttc atg aac gta gtc cag cga ctc aca ggg atc tcc tct ggt     288
Ser Glu Phe Met Asn Val Val Gln Arg Leu Thr Gly Ile Ser Ser Gly
                85                  90                  95

gtt ttc ctc gaa tct ggc ggc ggt gga gat gtt tca ccg gcg gcg agg     336
Val Phe Leu Glu Ser Gly Gly Gly Gly Asp Val Ser Pro Ala Ala Arg
            100                 105                 110

cta gcg tcc acg gaa aat gct agt cca aga gga gga aaa gaa ccg gct     384
Leu Ala Ser Thr Glu Asn Ala Ser Pro Arg Gly Gly Lys Glu Pro Ala
        115                 120                 125

gcg aga gat gag acg gtg gaa atc aac acg gct atg gaa gaa gca gct     432
Ala Arg Asp Glu Thr Val Glu Ile Asn Thr Ala Met Glu Glu Ala Ala
    130                 135                 140

gaa ttt ggt ggt tat gct ccg gga ata ctc tcg cca tct ccg gcc ttg     480
Glu Phe Gly Gly Tyr Ala Pro Gly Ile Leu Ser Pro Ser Pro Ala Leu
145                 150                 155                 160

ttg cca aca gct tct acc ggg ata ttc tct ccg atg tat cat caa ggt     528
Leu Pro Thr Ala Ser Thr Gly Ile Phe Ser Pro Met Tyr His Gln Gly
                165                 170                 175

ggg atg ttt tcg ccg gct ata cca ctg gga tta ttc tcg ccg gcg gga     576
```

```
Gly Met Phe Ser Pro Ala Ile Pro Leu Gly Leu Phe Ser Pro Ala Gly
            180                 185                 190

ttt atg agc ccg ttt cga agt cct ggc ttt act agt ttg gta gct tca      624
Phe Met Ser Pro Phe Arg Ser Pro Gly Phe Thr Ser Leu Val Ala Ser
        195                 200                 205

cca act ttt gct gat ttc ttt agt cat att tgg gat caa gat tag          669
Pro Thr Phe Ala Asp Phe Phe Ser His Ile Trp Asp Gln Asp
    210                 215                 220


<210>  2
<211>  222
<212>  PRT
<213>  Arabidopsis sp.

<400>  2

Met Asp Pro Ser Glu Tyr Phe Ala Gly Gly Asn Pro Ser Asp Gln Gln
1               5                   10                  15


Asn Gln Lys Arg Gln Leu Gln Ile Cys Gly Pro Arg Pro Ser Pro Leu
            20                  25                  30


Ser Val His Lys Asp Ser His Lys Ile Lys Lys Pro Pro Lys His Pro
        35                  40                  45


Ala Pro Pro Pro Asn Arg Asp Gln Pro Pro Pro Tyr Ile Pro Arg Glu
    50                  55                  60


Pro Val Val Ile Tyr Ala Val Ser Pro Lys Val Val His Ala Thr Ala
65                  70                  75                  80


Ser Glu Phe Met Asn Val Val Gln Arg Leu Thr Gly Ile Ser Ser Gly
                85                  90                  95


Val Phe Leu Glu Ser Gly Gly Gly Gly Asp Val Ser Pro Ala Ala Arg
            100                 105                 110


Leu Ala Ser Thr Glu Asn Ala Ser Pro Arg Gly Gly Lys Glu Pro Ala
        115                 120                 125


Ala Arg Asp Glu Thr Val Glu Ile Asn Thr Ala Met Glu Glu Ala Ala
    130                 135                 140


Glu Phe Gly Gly Tyr Ala Pro Gly Ile Leu Ser Pro Ser Pro Ala Leu
145                 150                 155                 160


Leu Pro Thr Ala Ser Thr Gly Ile Phe Ser Pro Met Tyr His Gln Gly
                165                 170                 175


Gly Met Phe Ser Pro Ala Ile Pro Leu Gly Leu Phe Ser Pro Ala Gly
            180                 185                 190


Phe Met Ser Pro Phe Arg Ser Pro Gly Phe Thr Ser Leu Val Ala Ser
        195                 200                 205
```

```
Pro Thr Phe Ala Asp Phe Phe Ser His Ile Trp Asp Gln Asp
    210                 215                 220


<210>  3
<211>  16
<212>  DNA
<213>  Arabidopsis sp.

<400>  3
atggatccgt cggagt                                                         16


<210>  4
<211>  16
<212>  DNA
<213>  Arabidopsis sp.

<400>  4
ctaatcttca tcccaa                                                         16


<210>  5
<211>  720
<212>  DNA
<213>  Arabidopsis sp.


<220>
<221>  CDS
<222>  (1)..(720)

<400>  5
atg gat aat aga tcg cca aga tca aga gga atc ttg ggt ccg aga cca          48
Met Asp Asn Arg Ser Pro Arg Ser Arg Gly Ile Leu Gly Pro Arg Pro
1               5                   10                  15

ata cca ttg aaa gtc cgt gga gat tcg cac aag atc atc aag aag cca          96
Ile Pro Leu Lys Val Arg Gly Asp Ser His Lys Ile Ile Lys Lys Pro
            20                  25                  30

cca cta gcg ccg cca cac ccg caa cca caa cca cca caa acc cat cag         144
Pro Leu Ala Pro Pro His Pro Gln Pro Gln Pro Pro Gln Thr His Gln
        35                  40                  45

caa gaa ccg tca caa tcg cgg ccg cca cct ggt ccc gtg att ata tac         192
Gln Glu Pro Ser Gln Ser Arg Pro Pro Pro Gly Pro Val Ile Ile Tyr
    50                  55                  60

aca gta tct ccc agg att atc cat aca cac cct aat aac ttc atg aca         240
Thr Val Ser Pro Arg Ile Ile His Thr His Pro Asn Asn Phe Met Thr
65                  70                  75                  80

ttg gtc caa cgt ctc aca ggt aaa acc tcc acc tcc aca aca tcc tcc         288
Leu Val Gln Arg Leu Thr Gly Lys Thr Ser Thr Ser Thr Thr Ser Ser
                85                  90                  95

tcc tat tct tca tct acg tca gca cca aaa gac gcg tca aca atg gtt         336
Ser Tyr Ser Ser Ser Thr Ser Ala Pro Lys Asp Ala Ser Thr Met Val
            100                 105                 110

gat aca tct cat ggg ttg ata tct ccg gcg gct cgg ttt gct gtt aca         384
Asp Thr Ser His Gly Leu Ile Ser Pro Ala Ala Arg Phe Ala Val Thr
        115                 120                 125

gag aag gct aat atc tca aac gaa cta ggg aca ttt gtt gga ggc gaa         432
Glu Lys Ala Asn Ile Ser Asn Glu Leu Gly Thr Phe Val Gly Gly Glu
```

130 135 140

ggg act atg gat caa tat tat cat tat cat cat cat cat cat cat caa 480
Gly Thr Met Asp Gln Tyr Tyr His Tyr His His His His His His Gln
145 150 155 160

gaa caa caa cat caa aat caa ggg ttc gag cgg cca agt ttc cac cat 528
Glu Gln Gln His Gln Asn Gln Gly Phe Glu Arg Pro Ser Phe His His
165 170 175

gct ggg att tta tcg ccg gga cct aat tct ctg ccg tcg gta tca ccg 576
Ala Gly Ile Leu Ser Pro Gly Pro Asn Ser Leu Pro Ser Val Ser Pro
180 185 190

gac ttc ttt tcc act att gga cca acc gat cca caa ggt ttt tcg tcg 624
Asp Phe Phe Ser Thr Ile Gly Pro Thr Asp Pro Gln Gly Phe Ser Ser
195 200 205

ttc ttt aat gac ttt aac tct atc ctt cag agt agt cca tcg aag att 672
Phe Phe Asn Asp Phe Asn Ser Ile Leu Gln Ser Ser Pro Ser Lys Ile
210 215 220

cag tct cct tct tct atg gac ctt ttc aac aat ttc ttt gat tct tga 720
Gln Ser Pro Ser Ser Met Asp Leu Phe Asn Asn Phe Phe Asp Ser
225 230 235

<210> 6
<211> 239
<212> PRT
<213> Arabidopsis sp.

<400> 6

Met Asp Asn Arg Ser Pro Arg Ser Arg Gly Ile Leu Gly Pro Arg Pro
1 5 10 15

Ile Pro Leu Lys Val Arg Gly Asp Ser His Lys Ile Ile Lys Lys Pro
20 25 30

Pro Leu Ala Pro Pro His Pro Gln Pro Gln Pro Pro Gln Thr His Gln
35 40 45

Gln Glu Pro Ser Gln Ser Arg Pro Pro Pro Gly Pro Val Ile Ile Tyr
50 55 60

Thr Val Ser Pro Arg Ile Ile His Thr His Pro Asn Asn Phe Met Thr
65 70 75 80

Leu Val Gln Arg Leu Thr Gly Lys Thr Ser Thr Ser Thr Thr Ser Ser
85 90 95

Ser Tyr Ser Ser Ser Thr Ser Ala Pro Lys Asp Ala Ser Thr Met Val
100 105 110

Asp Thr Ser His Gly Leu Ile Ser Pro Ala Ala Arg Phe Ala Val Thr
115 120 125

Glu Lys Ala Asn Ile Ser Asn Glu Leu Gly Thr Phe Val Gly Gly Glu
130 135 140

```
Gly Thr Met Asp Gln Tyr Tyr His Tyr His His His His His His Gln
145                 150                 155                 160

Glu Gln Gln His Gln Asn Gln Gly Phe Glu Arg Pro Ser Phe His His
                165                 170                 175

Ala Gly Ile Leu Ser Pro Gly Pro Asn Ser Leu Pro Ser Val Ser Pro
            180                 185                 190

Asp Phe Phe Ser Thr Ile Gly Pro Thr Asp Pro Gln Gly Phe Ser Ser
        195                 200                 205

Phe Phe Asn Asp Phe Asn Ser Ile Leu Gln Ser Ser Pro Ser Lys Ile
    210                 215                 220

Gln Ser Pro Ser Ser Met Asp Leu Phe Asn Asn Phe Phe Asp Ser
225                 230                 235


<210>  7
<211>  20
<212>  DNA
<213>  Arabidopsis sp.

<400>  7
atggataata gatcgccaag                                                   20


<210>  8
<211>  21
<212>  DNA
<213>  Arabidopsis sp.

<400>  8
tcaagaatca aagaaattgt t                                                 21


<210>  9
<211>  791
<212>  DNA
<213>  Brassica oleracea


<220>
<221>  CDS
<222>  (139)..(789)

<400>  9
taatttttcc cttttttttt tgtttataaa tgttttggtc aatactagct cgtcgtcgac      60

aaagattcat ttcgattccc aaaccacaca agaagaacac aaattagctc gaaagaaaca     120

aactcttttg agaaaata atg gat ccg tcg gag tct ttc gcc ggc ggc aat       171
                    Met Asp Pro Ser Glu Ser Phe Ala Gly Gly Asn
                    1               5                   10

cct tcc gac caa cag aac cag aaa cgt cag ctt cag atc tgt ggt cct       219
Pro Ser Asp Gln Gln Asn Gln Lys Arg Gln Leu Gln Ile Cys Gly Pro
            15                  20                  25

cgt ccc tca cct ctc agc gtc aac aaa gac tct cac aag atc aag aaa       267
Arg Pro Ser Pro Leu Ser Val Asn Lys Asp Ser His Lys Ile Lys Lys
        30                  35                  40
```

```
cct cct aaa cac cct gct cct ccg cct cag cat cgc gac caa gct ccg      315
Pro Pro Lys His Pro Ala Pro Pro Pro Gln His Arg Asp Gln Ala Pro
    45                  50                  55

ctc tac gct gct cga gag ccg gtg gtc atc tac gcc gtc tcg ccg aaa      363
Leu Tyr Ala Ala Arg Glu Pro Val Val Ile Tyr Ala Val Ser Pro Lys
60                  65                  70                  75

gtc gtc cac acc aca gcc tcg gat ttc atg aac gtc gtc cag cgt ctc      411
Val Val His Thr Thr Ala Ser Asp Phe Met Asn Val Val Gln Arg Leu
                80                  85                  90

acc ggc atc tca tcc gcc gtc ttc ctc gaa tcc ggt aac ggc gga gat      459
Thr Gly Ile Ser Ser Ala Val Phe Leu Glu Ser Gly Asn Gly Gly Asp
            95                  100                 105

gta tct ccg gcg gcg aga ctc gcc gcg acc gag aat gca agc ccg aga      507
Val Ser Pro Ala Ala Arg Leu Ala Ala Thr Glu Asn Ala Ser Pro Arg
        110                 115                 120

gga gga aaa gaa ccg gtg atg gcg gct aaa gat gag acg gtg gaa atc      555
Gly Gly Lys Glu Pro Val Met Ala Ala Lys Asp Glu Thr Val Glu Ile
    125                 130                 135

gcg acg gct atg gaa gaa gca gcc gag ttg agc ggc tat gcg ccg ggg      603
Ala Thr Ala Met Glu Glu Ala Ala Glu Leu Ser Gly Tyr Ala Pro Gly
140                 145                 150                 155

ata ctc tcc cct tct ccg gct atg tta ccg aca gct tct gcc gga ata      651
Ile Leu Ser Pro Ser Pro Ala Met Leu Pro Thr Ala Ser Ala Gly Ile
                160                 165                 170

ttc tcg cag atg act act cac caa ggt ggg atg ttc tcg ccg gga ttg      699
Phe Ser Gln Met Thr Thr His Gln Gly Gly Met Phe Ser Pro Gly Leu
            175                 180                 185

ttt tcg ccg gcg ggg tta atg agc ccg ttt ggt ttt gct agc ttg gtt      747
Phe Ser Pro Ala Gly Leu Met Ser Pro Phe Gly Phe Ala Ser Leu Val
        190                 195                 200

gct tct cca acg ttt gct gat ttg ttc agt cat att tgg gga ta           791
Ala Ser Pro Thr Phe Ala Asp Leu Phe Ser His Ile Trp Gly
    205                 210                 215


<210>  10
<211>  217
<212>  PRT
<213>  Brassica oleracea

<400>  10

Met Asp Pro Ser Glu Ser Phe Ala Gly Gly Asn Pro Ser Asp Gln Gln
1               5                   10                  15

Asn Gln Lys Arg Gln Leu Gln Ile Cys Gly Pro Arg Pro Ser Pro Leu
            20                  25                  30

Ser Val Asn Lys Asp Ser His Lys Ile Lys Lys Pro Pro Lys His Pro
        35                  40                  45

Ala Pro Pro Pro Gln His Arg Asp Gln Ala Pro Leu Tyr Ala Ala Arg
    50                  55                  60
```

Glu Pro Val Val Ile Tyr Ala Val Ser Pro Lys Val Val His Thr Thr
65 70 75 80

Ala Ser Asp Phe Met Asn Val Val Gln Arg Leu Thr Gly Ile Ser Ser
85 90 95

Ala Val Phe Leu Glu Ser Gly Asn Gly Gly Asp Val Ser Pro Ala Ala
100 105 110

Arg Leu Ala Ala Thr Glu Asn Ala Ser Pro Arg Gly Gly Lys Glu Pro
115 120 125

Val Met Ala Ala Lys Asp Glu Thr Val Glu Ile Ala Thr Ala Met Glu
130 135 140

Glu Ala Ala Glu Leu Ser Gly Tyr Ala Pro Gly Ile Leu Ser Pro Ser
145 150 155 160

Pro Ala Met Leu Pro Thr Ala Ser Ala Gly Ile Phe Ser Gln Met Thr
165 170 175

Thr His Gln Gly Gly Met Phe Ser Pro Gly Leu Phe Ser Pro Ala Gly
180 185 190

Leu Met Ser Pro Phe Gly Phe Ala Ser Leu Val Ala Ser Pro Thr Phe
195 200 205

Ala Asp Leu Phe Ser His Ile Trp Gly
210 215

<210> 11
<211> 20
<212> DNA
<213> Brassica oleracea

<400> 11
atggatccgt cggagtcttt 20

<210> 12
<211> 20
<212> DNA
<213> Brassica oleracea

<400> 12
tatccccaaa tatgactgaa 20

<210> 13
<211> 878
<212> DNA
<213> Brassica oleracea

<220>
<221> CDS
<222> (198)..(833)

<400> 13

aaaagtcaac attttgaaag tcaaactaat cggtctcaga aaacaaaaat aactttgtgt 60

gttgatgttt aggtcaatat actcgtcgtc aaaacatccc ttcaatttct cagaccaaac 120

acagagaaga aacaagttgg atccaaactc tctacaacaa aaagtagtga acgagagaag 180

ctctccccaa gcgttta atg gat ccg tcg gag cac ttc gcc ggc ggt aat 230
Met Asp Pro Ser Glu His Phe Ala Gly Gly Asn
1 5 10

cct ttc gat caa cag act cca aaa cgt cag ctt cag atc tgt ggc cct 278
Pro Phe Asp Gln Gln Thr Pro Lys Arg Gln Leu Gln Ile Cys Gly Pro
15 20 25

cgt cct tca cct cta agc gtc aac aaa gac tct cac aag atc aag aaa 326
Arg Pro Ser Pro Leu Ser Val Asn Lys Asp Ser His Lys Ile Lys Lys
30 35 40

cct ccc agg cac cct gct cca cct cct cag cat cac cgc gac caa gct 374
Pro Pro Arg His Pro Ala Pro Pro Pro Gln His His Arg Asp Gln Ala
45 50 55

ccg ctc tac cct cct cga gag ccg gtg gtt atc tac gcc gtc tcg ccg 422
Pro Leu Tyr Pro Pro Arg Glu Pro Val Val Ile Tyr Ala Val Ser Pro
60 65 70 75

aaa gtc gtg cac acc aca acc tcc gat ttc atg aac gtc gtc cag cgt 470
Lys Val Val His Thr Thr Thr Ser Asp Phe Met Asn Val Val Gln Arg
80 85 90

ctc acc ggg atc tcc tcc gag gtc ttc ctc gaa tca aga aac gac gga 518
Leu Thr Gly Ile Ser Ser Glu Val Phe Leu Glu Ser Arg Asn Asp Gly
95 100 105

gat gta tca ccg gcg gcg aga ctc gcc gcg acg gag aat gct agc ccg 566
Asp Val Ser Pro Ala Ala Arg Leu Ala Ala Thr Glu Asn Ala Ser Pro
110 115 120

aga gga gga aag gaa ccg gtg gaa agc tcg acg gct atg gaa gaa gca 614
Arg Gly Gly Lys Glu Pro Val Glu Ser Ser Thr Ala Met Glu Glu Ala
125 130 135

gct gag ttc ggt tgt tat gtg ccg gga ata ctc tcg ccg tct ccg gct 662
Ala Glu Phe Gly Cys Tyr Val Pro Gly Ile Leu Ser Pro Ser Pro Ala
140 145 150 155

atg tta ccg acc gtt ccc gcc gga att ttc tct ccg atg ttt cac cta 710
Met Leu Pro Thr Val Pro Ala Gly Ile Phe Ser Pro Met Phe His Leu
160 165 170

ggt ggg ttg ttt tcg ccg gcg ttg ccg ccg gga tta ttt tcg ccg gca 758
Gly Gly Leu Phe Ser Pro Ala Leu Pro Pro Gly Leu Phe Ser Pro Ala
175 180 185

gga tta atg agc cct ggt tat gct agt ttg gcg tca cca aat ttt gct 806
Gly Leu Met Ser Pro Gly Tyr Ala Ser Leu Ala Ser Pro Asn Phe Ala
190 195 200

gat ttc ttc agt cac att tgg gat cct tagagaatag attattagtt 853
Asp Phe Phe Ser His Ile Trp Asp Pro
205 210

tttttatta tttacatttt atgta 878

<210> 14
<211> 212
<212> PRT
<213> Brassica oleracea

<400> 14

Met Asp Pro Ser Glu His Phe Ala Gly Gly Asn Pro Phe Asp Gln Gln
1 5 10 15

Thr Pro Lys Arg Gln Leu Gln Ile Cys Gly Pro Arg Pro Ser Pro Leu
20 25 30

Ser Val Asn Lys Asp Ser His Lys Ile Lys Lys Pro Pro Arg His Pro
35 40 45

Ala Pro Pro Pro Gln His His Arg Asp Gln Ala Pro Leu Tyr Pro Pro
50 55 60

Arg Glu Pro Val Val Ile Tyr Ala Val Ser Pro Lys Val Val His Thr
65 70 75 80

Thr Thr Ser Asp Phe Met Asn Val Val Gln Arg Leu Thr Gly Ile Ser
85 90 95

Ser Glu Val Phe Leu Glu Ser Arg Asn Asp Gly Asp Val Ser Pro Ala
100 105 110

Ala Arg Leu Ala Ala Thr Glu Asn Ala Ser Pro Arg Gly Gly Lys Glu
115 120 125

Pro Val Glu Ser Ser Thr Ala Met Glu Glu Ala Ala Glu Phe Gly Cys
130 135 140

Tyr Val Pro Gly Ile Leu Ser Pro Ser Pro Ala Met Leu Pro Thr Val
145 150 155 160

Pro Ala Gly Ile Phe Ser Pro Met Phe His Leu Gly Gly Leu Phe Ser
165 170 175

Pro Ala Leu Pro Pro Gly Leu Phe Ser Pro Ala Gly Leu Met Ser Pro
180 185 190

Gly Tyr Ala Ser Leu Ala Ser Pro Asn Phe Ala Asp Phe Phe Ser His
195 200 205

Ile Trp Asp Pro
210

<210> 15
<211> 393
<212> DNA
<213> Glycine max

<220>
<221> CDS
<222> (1)..(393)

<400> 15

```
caa ctt caa ggt cca cgc cct aca cct ctc aga ata aac aaa gac tct       48
Gln Leu Gln Gly Pro Arg Pro Thr Pro Leu Arg Ile Asn Lys Asp Ser
1               5                   10                  15

cat aaa atc aag aaa cca ccg ttg gca cca caa cct tca cac cct cat       96
His Lys Ile Lys Lys Pro Pro Leu Ala Pro Gln Pro Ser His Pro His
            20                  25                  30

caa cct cca ccg cgc caa cct ata ata atc tac acc gtg tcc ccc aag      144
Gln Pro Pro Pro Arg Gln Pro Ile Ile Ile Tyr Thr Val Ser Pro Lys
        35                  40                  45

gtg att cac acc acc cca agt gac ttc atg aac ctc gtc caa cgc ctc      192
Val Ile His Thr Thr Pro Ser Asp Phe Met Asn Leu Val Gln Arg Leu
    50                  55                  60

act ggg tcc agt tct tct tcc tct gct gaa gtg gtc atg tcc aac aat      240
Thr Gly Ser Ser Ser Ser Ser Ser Ala Glu Val Val Met Ser Asn Asn
65                  70                  75                  80

aac aac acc act cat gtc gac cct ttc aac aac ggc ggc ggc gga atg      288
Asn Asn Thr Thr His Val Asp Pro Phe Asn Asn Gly Gly Gly Gly Met
                85                  90                  95

gtg tcg ccg gcg gcg cgt tac gcc acc ata gag aag gcc atg tcc cct      336
Val Ser Pro Ala Ala Arg Tyr Ala Thr Ile Glu Lys Ala Met Ser Pro
            100                 105                 110

atg ggg aaa aaa cat gtt ctt ctt cca agt gtg aac aat att ata agc      384
Met Gly Lys Lys His Val Leu Leu Pro Ser Val Asn Asn Ile Ile Ser
        115                 120                 125

gat gtg gaa                                                          393
Asp Val Glu
    130
```

<210> 16
<211> 131
<212> PRT
<213> Glycine max

<400> 16

```
Gln Leu Gln Gly Pro Arg Pro Thr Pro Leu Arg Ile Asn Lys Asp Ser
1               5                   10                  15

His Lys Ile Lys Lys Pro Pro Leu Ala Pro Gln Pro Ser His Pro His
            20                  25                  30

Gln Pro Pro Pro Arg Gln Pro Ile Ile Ile Tyr Thr Val Ser Pro Lys
        35                  40                  45

Val Ile His Thr Thr Pro Ser Asp Phe Met Asn Leu Val Gln Arg Leu
    50                  55                  60

Thr Gly Ser Ser Ser Ser Ser Ser Ala Glu Val Val Met Ser Asn Asn
65                  70                  75                  80

Asn Asn Thr Thr His Val Asp Pro Phe Asn Asn Gly Gly Gly Gly Met
                85                  90                  95
```

Val Ser Pro Ala Ala Arg Tyr Ala Thr Ile Glu Lys Ala Met Ser Pro
100 105 110

Met Gly Lys Lys His Val Leu Leu Pro Ser Val Asn Asn Ile Ile Ser
115 120 125

Asp Val Glu
130

<210> 17
<211> 19
<212> DNA
<213> Glycine max

<400> 17
ccatagagaa ggccatgtc 19

<210> 18
<211> 20
<212> DNA
<213> Glycine max

<400> 18
tgaatgttgt ggtgccaacg 20

<210> 19
<211> 927
<212> DNA
<213> Oryza sp.

<220>
<221> CDS
<222> (7)..(927)

<400> 19
gtggcg atg gaa ttc ccg tcg tcg acg tcg ccg tcg ccg tcg ccg tcg 48
Met Glu Phe Pro Ser Ser Thr Ser Pro Ser Pro Ser Pro Ser
1 5 10

tcc ggg cag cat cag cag cag ccg acg acg ccg cgg cgg cag ctt cag 96
Ser Gly Gln His Gln Gln Gln Pro Thr Thr Pro Arg Arg Gln Leu Gln
15 20 25 30

ggc ccg cgc ccc ccg cgg ctc aac gtg cgg atg gag tcg cac gcc atc 144
Gly Pro Arg Pro Pro Arg Leu Asn Val Arg Met Glu Ser His Ala Ile
35 40 45

aag aag ccg tcg tcc ggg gcg gcc gcg gcg gcg gcg gcg gcg cag gcg 192
Lys Lys Pro Ser Ser Gly Ala Ala Ala Ala Ala Ala Ala Ala Gln Ala
50 55 60

agg cgg gag cag cag cag ccg ccg ccg cgg gcg ccg gtg atc atc tac 240
Arg Arg Glu Gln Gln Gln Pro Pro Pro Arg Ala Pro Val Ile Ile Tyr
65 70 75

gac gcg tcg ccg aag att atc cac gcc aag ccc aac gag ttc atg gcg 288
Asp Ala Ser Pro Lys Ile Ile His Ala Lys Pro Asn Glu Phe Met Ala
80 85 90

ctc gtg cag cgg ctc acc ggc ccg ggg tcg ggg ccg ccg gcg ccg ccg 336
Leu Val Gln Arg Leu Thr Gly Pro Gly Ser Gly Pro Pro Ala Pro Pro
95 100 105 110

```
cat caa ggg gag gcc cag gcg cag gac tac ccg atg atg gac gag gcc        384
His Gln Gly Glu Ala Gln Ala Gln Asp Tyr Pro Met Met Asp Glu Ala
                115                 120                 125

gcc gcg cag cag ttc ttc ccg ccg gag ctg ctg ctc tcg ccg tcg gcc        432
Ala Ala Gln Gln Phe Phe Pro Pro Glu Leu Leu Leu Ser Pro Ser Ala
            130                 135                 140

gcg atg tcc ccg gcg gcg agg ctg gcg acc atc gag agg tcc gtc cgc        480
Ala Met Ser Pro Ala Ala Arg Leu Ala Thr Ile Glu Arg Ser Val Arg
        145                 150                 155

ccg atg ccc gag ccg gcg ccg gag tac gtg gac atc acg aac ggc ggc        528
Pro Met Pro Glu Pro Ala Pro Glu Tyr Val Asp Ile Thr Asn Gly Gly
    160                 165                 170

ggc ggc ggc ggg gtc gac gac ggc ggc ctc gcg gcg atc ctc ggc tcg        576
Gly Gly Gly Gly Val Asp Asp Gly Gly Leu Ala Ala Ile Leu Gly Ser
175                 180                 185                 190

atc cgg cca ggc atc ctc tcc ccg ctc ccc tcc tcc ctc ccg ccc gcc        624
Ile Arg Pro Gly Ile Leu Ser Pro Leu Pro Ser Ser Leu Pro Pro Ala
                195                 200                 205

gcc gtc ccc ggc cag ttc tcg ccg ctc ccg ttc gac gcg agg ccg ctc        672
Ala Val Pro Gly Gln Phe Ser Pro Leu Pro Phe Asp Ala Arg Pro Leu
            210                 215                 220

ccg ttc gac gcg agc tgc atc agc tgg ctc aac gag ctg agc ccc atc        720
Pro Phe Asp Ala Ser Cys Ile Ser Trp Leu Asn Glu Leu Ser Pro Ile
        225                 230                 235

ctc cgg gcc gcc tcc gcc ggc gcg gcc tcg tcc ggc agc ggc ggc ggc        768
Leu Arg Ala Ala Ser Ala Gly Ala Ala Ser Ser Gly Ser Gly Gly Gly
    240                 245                 250

ggc agc ggt ggc aac acc agc aac ggc ggc ggc gcc cgc ccg ccg ccg        816
Gly Ser Gly Gly Asn Thr Ser Asn Gly Gly Gly Ala Arg Pro Pro Pro
255                 260                 265                 270

tcc tac tac gcc gac cca ttc gtc ccc agc cca cgt cac ctc ctc gcc        864
Ser Tyr Tyr Ala Asp Pro Phe Val Pro Ser Pro Arg His Leu Leu Ala
                275                 280                 285

acg ccc acc gtg ccg tcg ccg gcg acc tgc gcc gag ctc ttc agc aac        912
Thr Pro Thr Val Pro Ser Pro Ala Thr Cys Ala Glu Leu Phe Ser Asn
            290                 295                 300

ctg ccg gat ctc tag                                                    927
Leu Pro Asp Leu
        305
```

<210>   20
<211>   306
<212>   PRT
<213>   Oryza sp.

<400>   20

```
Met Glu Phe Pro Ser Ser Thr Ser Pro Ser Pro Ser Pro Ser Ser Gly
1               5                   10                  15

Gln His Gln Gln Gln Pro Thr Thr Pro Arg Arg Gln Leu Gln Gly Pro
            20                  25                  30

Arg Pro Pro Arg Leu Asn Val Arg Met Glu Ser His Ala Ile Lys Lys
```

35 40 45

Pro Ser Ser Gly Ala Ala Ala Ala Ala Ala Ala Ala Gln Ala Arg Arg
50 55 60

Glu Gln Gln Gln Pro Pro Pro Arg Ala Pro Val Ile Ile Tyr Asp Ala
65 70 75 80

Ser Pro Lys Ile Ile His Ala Lys Pro Asn Glu Phe Met Ala Leu Val
85 90 95

Gln Arg Leu Thr Gly Pro Gly Ser Gly Pro Pro Ala Pro Pro His Gln
100 105 110

Gly Glu Ala Gln Ala Gln Asp Tyr Pro Met Met Asp Glu Ala Ala Ala
115 120 125

Gln Gln Phe Phe Pro Pro Glu Leu Leu Leu Ser Pro Ser Ala Ala Met
130 135 140

Ser Pro Ala Ala Arg Leu Ala Thr Ile Glu Arg Ser Val Arg Pro Met
145 150 155 160

Pro Glu Pro Ala Pro Glu Tyr Val Asp Ile Thr Asn Gly Gly Gly Gly
165 170 175

Gly Gly Val Asp Asp Gly Gly Leu Ala Ala Ile Leu Gly Ser Ile Arg
180 185 190

Pro Gly Ile Leu Ser Pro Leu Pro Ser Ser Leu Pro Pro Ala Ala Val
195 200 205

Pro Gly Gln Phe Ser Pro Leu Pro Phe Asp Ala Arg Pro Leu Pro Phe
210 215 220

Asp Ala Ser Cys Ile Ser Trp Leu Asn Glu Leu Ser Pro Ile Leu Arg
225 230 235 240

Ala Ala Ser Ala Gly Ala Ala Ser Ser Gly Ser Gly Gly Gly Gly Ser
245 250 255

Gly Gly Asn Thr Ser Asn Gly Gly Gly Ala Arg Pro Pro Pro Ser Tyr
260 265 270

Tyr Ala Asp Pro Phe Val Pro Ser Pro Arg His Leu Leu Ala Thr Pro
275 280 285

Thr Val Pro Ser Pro Ala Thr Cys Ala Glu Leu Phe Ser Asn Leu Pro
290 295 300

Asp Leu
305

<210> 21
<211> 16
<212> DNA
<213> Oryza sp.

<400> 21
atggaattcc cgtcgt 16

<210> 22
<211> 19
<212> DNA
<213> Oryza sp.

<400> 22
ctagagatcc ggcaggttg 19

<210> 23
<211> 781
<212> DNA
<213> CaMV 35S promoter duplicated

<400> 23
atggtggagc acgacactct cgtctactcc aagaatatca aagatacagt ctcagaagac 60

caaagggcta ttgagacttt tcaacaaagg gtaatatcgg gaaacctcct cggattccat 120

tgcccagcta tctgtcactt catcaaaagg acagtagaaa aggaaggtgg cacctacaaa 180

tgccatcatt gcgataaagg aaaggctatc gttcaagatg cctctgccga cagtggtccc 240

aaagatggac ccccacccac gaggagcatc gtggaaaaag aagacgttcc aaccacgtct 300

tcaaagcaag tggattgatg tgataacatg gtggagcacg acactctcgt ctactccaag 360

aatatcaaag atacagtctc agaagaccaa agggctattg agacttttca acaaagggta 420

atatcgggaa acctcctcgg attccattgc ccagctatct gtcacttcat caaaaggaca 480

gtagaaaagg aaggtggcac ctacaaatgc catcattgcg ataaaggaaa ggctatcgtt 540

caagatgcct ctgccgacag tggtcccaaa gatggacccc cacccacgag gagcatcgtg 600

gaaaaagaag acgttccaac cacgtcttca aagcaagtgg attgatgtga tatctccact 660

gacgtaaggg atgacgcaca atcccactat ccttcgcaag acccttcctct atataaggaa 720

gttcatttca tttggagagg acacgctgaa atcaccagtc tctctctaca aatctatctc 780

t 781

<210> 24
<211> 253
<212> DNA
<213> Agrobacterium NOS terminator

<400> 24
cgttcaaaca tttggcaata aagtttctta agattgaatc ctgttgccgg tcttgcgatg 60

attatcatat aatttctgtt gaattacgtt aagcatgtaa taattaacat gtaatgcatg 120

acgttattta tgagatgggt ttttatgatt agagtcccgc aattatacat ttaatacgcg 180

atagaaaaca aaatatagcg cgcaaactag gataaattat cgcgcgcggt gtcatctatg 240

ttactagatc ggg 253

<210> 25
<211> 189
<212> DNA
<213> Synthetic intron

<400> 25
gtaagtttct gcttctacct ttgatatata tataataatt atcattaatt agtagtaata 60

taatatttca aatatttttt tcaaaataaa agaatgtagt atatagcaat tgcttttctg 120

tagtttataa gtgtgtatat tttaatttat aacttttcta atatatgacc aaaatttgtt 180

gatgtgcag 189

<210> 26
<211> 207
<212> PRT
<213> Oryza sp.

<400> 26

Met Glu Gln Gln Leu Ser Ser Pro Ser Ala Ser Gln Arg Gly Gly Gly
1 5 10 15

Arg Glu Leu Gln Gly Pro Arg Pro Ala Pro Leu Lys Val Arg Lys Glu
20 25 30

Ser His Lys Ile Arg Lys Gln Glu Pro Val Gln Gln Leu Arg Gln Pro
35 40 45

Val Ile Ile Tyr Thr Met Ser Pro Lys Val Val His Ala Asn Ala Ala
50 55 60

Asp Phe Met Ser Val Val Gln Arg Leu Thr Gly Ala Pro Pro Thr Ala
65 70 75 80

Pro Pro Gln Pro Gln Pro His His Pro Thr Leu Leu Ala Gln Met Pro
85 90 95

Pro Gln Pro Ser Phe Pro Phe His Leu Gln Gln Gln Asp Ala Trp Pro
100 105 110

Gln Gln Gln His Ser Pro Ala Ala Ile Glu Gln Ala Ala Ala Arg Ser
115 120 125

Ser Gly Ala Asp Leu Pro Pro Leu Pro Ser Ile Leu Ser Pro Val Pro
130 135 140

Gly Thr Val Leu Pro Ala Ile Pro Ala Ser Phe Phe Ser Pro Pro Ser
145 150 155 160

Leu Ile Ser Pro Val Pro Phe Leu Gly Ala Thr Thr Thr Ser Ser Ala
165 170 175

Ala Pro Ser Thr Ser Pro Ser Pro Met Gly Gly Ser Ala Tyr Tyr Trp
            180   185   190

Asp Leu Phe Asn Met Gln Gln Gln Gln His Tyr His His Gln Asn
        195   200   205

<210> 27
<211> 238
<212> PRT
<213> Zea mays

<400> 27

Met Asp Pro Pro Ser Ser Ser Gly Arg Pro Thr Thr Pro Arg Arg Gln
1   5   10   15

Leu Gln Gly Pro Arg Pro Pro Arg Leu Asn Val Arg Met Glu Ser His
            20   25   30

Ala Ile Lys Lys Pro Ser Ala Ser Gly Ala Pro Pro Ala Pro Gly Gln
        35   40   45

Gly Arg Pro Arg Asp His His His His His Pro Gln Pro Gly Arg Ala
    50   55   60

Pro Val Ile Ile Tyr Asp Ala Ser Pro Lys Val Ile His Ala Lys Pro
65   70   75   80

Ser Glu Phe Met Ala Leu Val Gln Arg Leu Thr Gly Pro Gly Ala Gln
                85   90   95

Ala Gln His Glu Arg His Val Ala Asp Asp Asp Ala Thr Ala Asn Gly
            100   105   110

Gly Gly Val Leu Gly Gln Ala Phe Leu Pro Pro Glu Leu Leu Leu Ser
        115   120   125

Pro Ser Ala Ala Met Ser Pro Ala Ala Arg Leu Ala Thr Ile Glu Arg
    130   135   140

Ser Val Arg Pro Val Pro Ala Pro Ala Pro Ala Pro Asp Tyr Ala Ala
145   150   155   160

Asp Gly His Pro Arg Gly Gly Ala Arg Pro Arg Glu Ala Pro Arg His
                165   170   175

Pro Val Pro Ala Ala Val Leu Ala Ala Ala Gly Arg Arg Val Gly Pro
            180   185   190

Val Leu Ala Ala Ala Leu Arg Pro Gln Gln Arg Gln Leu Ala Gln Arg
        195   200   205

Ala Gln Pro His Pro Pro Gly Ser Val His Gly Gln Arg Ser Ala Pro
    210   215   220

Leu Ala His Ala His Gly Pro Thr Gly Gly Ser Arg Gln Pro
225 230 235

<210> 28
<211> 271
<212> PRT
<213> Zea mays

<400> 28

Gln Gly Pro Arg Pro Pro Arg Leu Ala Val Ser Lys Asp Ser His Lys
1 5 10 15

Val Arg Lys Pro Pro Val Ala Pro Gln Arg Gln Gln His Gln His Gln
20 25 30

Gln Pro Ala Ala Gln Leu Gln Gln Gln Gln His Gln Tyr His Gln Gln
35 40 45

Gln Gln Gln Gln Gly Arg Gln Pro Val Ile Ile Tyr Asp Ala Ser Pro
50 55 60

Lys Val Ile His Thr Lys Pro Gly Asp Phe Met Ala Leu Val Gln Arg
65 70 75 80

Leu Thr Gly Pro Gly Ser Thr Ser Gln Ala Gln Phe Asp Ala Ala Ala
85 90 95

Ala Ala Ala Gly Pro Ser His Pro Ala Ala Met Glu Phe Glu Pro Arg
100 105 110

Glu Phe Leu Leu Ser Pro Thr Ala Ala Leu Ser Pro Ala Ala Arg Leu
115 120 125

Ala Ala Ile Glu Arg Ser Val Arg Pro Leu Pro Pro His His Ala Pro
130 135 140

Ala Ala Val Pro Pro Tyr Phe Gly Ala Thr Asn Asp Asp Gly Phe Phe
145 150 155 160

Leu Pro Gly Ser Ala Asp Met Asp Ser Leu Ser Ala Ala Leu Gly Pro
165 170 175

Pro Ala Gly Arg Pro Gly Ile Leu Ser Pro Ala Ala Leu Pro Pro Ala
180 185 190

Ala Ser Thr Gly Leu Phe Ser Pro Met Pro Phe Asp Pro Ser Cys Leu
195 200 205

Ser Trp Leu Ser Glu Leu Ser Pro Phe Leu Pro Ser Ala Gly Thr Arg
210 215 220

Ala Ala Ala Ala Gly Leu Leu Asp Gln Ala Pro Phe Ala Pro Ser Pro
225   230   235   240

Arg Ser Ser Leu Leu Leu Ser Thr Pro Thr Met Pro Ser Pro Ala Thr
                245   250   255

Phe Ser Val Leu Glu Phe Phe Ser Ser Pro Asn Phe Pro Asp Leu
            260   265   270